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14. ABSTRACT

Prostate cancer (PCa) remains to be the most common non-skin cancer in the US. Currently available screening tests for PCa including prostate specific antigen (PSA) test, digital rectal examination (DRE) and prostate biopsy, call for more accurate and non-invasive techniques to detect, diagnose, and stratify the disease based on molecular markers present in the body fluids. There has been an impressive emergence of mass spectrometry based technologies applied toward the study of such biomolecular markers of disease states. Our focus on utilization of such techniques towards prostate cancer will promise a better health and future for PCa patients. We have devised strategies to isolate and identify protein biomarkers from PCa patients in the clinical gray-area where PSA fails to detect cancer. Identification of such cancer biomarkers will assist in development of better non-invasive diagnostic tools for prostate cancer and may also lead to better therapeutic targets.

15. SUBJECT TERMS
MALDI-TOF MS, Prostate Cancer, Biomarkers

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1. Introduction

A large number of proteins that are relevant in understanding the biological processes are expressed at low levels in the system. Therefore, there is a need for highly sensitive, high throughput methods to analyze a wide dynamic range of proteins.
Our group has demonstrated that high-throughput proteomic approaches for "protein profiling" has tremendous potential for identifying biomarkers to improve cancer diagnosis (Adam et al. 2002, Qu et al. 2002, Malik et al., 2005).

In order to improve the ability to "mine" the full depth of the proteome, Bruker Daltonics has newly introduced the UltraFlex™ MALDI-TOF/TOF instrument equipped with ClinProt, a robotic bead-based sample processing station. The instrument provides improved ability to mine deeper into the proteome, improved resolution/accuracy and the ability to achieve peptide/protein identification (Suckau et al. 2003).

2. The **Specific Aims** of our original application were-

AIM 1. Serum cohort to identify prostate cancer (PCa) population with minimal clinical symptoms.

AIM 2. Discovery of protein biomarkers for the early detection of PCa in cohort.

AIM 3. Isolation and identification of the protein biomarkers.

AIM 4. Development of MS-assisted immunoassay for PCa diagnostics.

3. Key Research Accomplishments

3.A. Serum cohort to identify prostate cancer (PCa) population with minimal clinical symptoms. Our studies are directed at the male population that present with marginal symptoms (such as low PSA levels and/or positive DRE) and who undergo biopsy. We have identified and collected serum specimens from 185 patients with positive prostate biopsy. We have also collected a set of 223 serum samples from patients with negative biopsy. The samples are stored at -70^{o}C in small aliquots ready to be used for this study.

Only pretreatment samples, obtained at the time of diagnosis of prostate cancer, were collected for use in this study. All samples were obtained from properly consented patients through the institutional review board approved protocols.

3.B. Serum protein profiling for PCa diagnostics. In our original application, we proposed to establish the clinical utility of high-throughput proteomic approaches to protein profiling. In addition, we also proposed to extend the proof-of-concept of the utility of mass spectrometry based approaches to specific early detection objectives in PCa by applying these proven approaches to the characterization and sequence identification of promising biomarkers for detecting early cancer.

Since, the proteomes of complex body fluids like serum have biomarkers spread over a wide range of concentrations (Semmes et al. 2006), the key to biomarker discovery lies in effective sample preparation prior to MS-based analyses. We therefore spent a significant portion of our efforts in optimizing the strategies to enrich low abundant proteins to allow for their identification by mass spectrometry. To achieve this objective, we have been developing methodologies for up-front fractionation of serum for MALDI profiling. Initially we assessed two parameters, which fractionation strategy provides the most differential capture of proteins between case and control, and whether serum depletion prior to fractionation improves the detection of these proteins. We utilized the magnetic bead-based approaches of fractionation for automation of the techniques on the ClinProt robotic workstation. The employment of functionalized magnetic bead based techniques in conjunction with mass spectrometry combines short processing times and automatic workflows with high-resolution analyses.

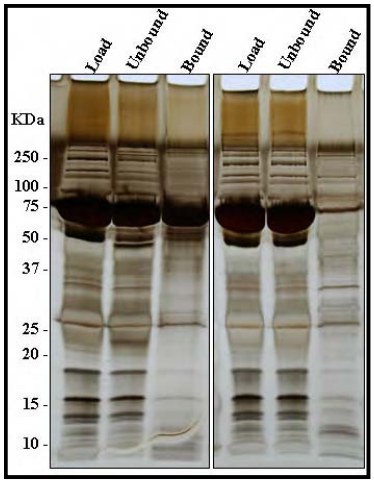

Figure 1. Proteins from quality control (QC) serum were captured by IMAC (left) or ConA lectin (right) magnetic beads. Neat serum (load), flow-through (unbound) and eluate (bound) fractions were analyzed by SDS-PAGE.

One of our approaches utilized the application of lectin capture to enrich for glycosylated forms of proteins in sera from PCa subjects. The lectin affinity approach offers several distinct biochemical advantages toward protein fractionation. Glycosylation is with at least 50% the most common form of post-translational modification of proteins and the degree and type of glycosylation depend on the status of cells, thus linking them to certain diseases (Dube and Bertozzi 2005). Besides, many major serum proteins, in particular albumin, are not glycosylated and are therefore not bound efficiently by lectins, offering a concomitant decrease in complexity of the serum protein targets (**Figure 1**). There are many lectin types available commercially in pure, bead-based forms which allows for automation of assays in the front-end to mass spectrometry and thereby facilitating high through-put analyses (Sparbier et al. 2005, 2006).

The biological affinity of lectins offers multiple uses and strategies to maximize the information gained from precious clinical samples. Our group has demonstrated in a proof-of-concept study, the specific enrichment of glycosylated peptides and proteins from PCa sera by lectin affinity chromatography supported by functionalized magnetic particles. I am a co-author in this research that has been published this year (Drake et al. 2006). Reprint of the article has been attached at the end of this report.

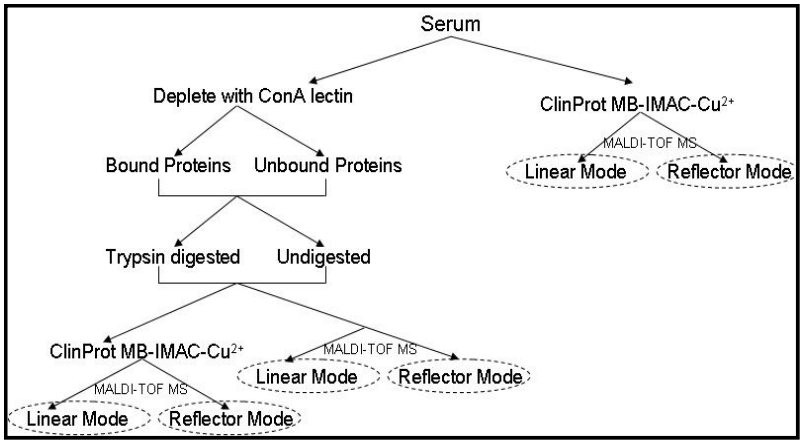

Figure 2. Experimental flow chart for protein profiling using mass spectrometry.

Our approach to utilize the lectin capture strategy for high-throughput serum expression profiling is summarized in **figure 2**. This involves capture of the intact glycoproteins by lectins, followed by trypsin digestion to generate peptides and final affinity separation of peptides prior to MALDI-TOF and/or tandem mass spectrometry analyses. All of these steps can be fully automated using bead-based supports coupled to liquid sample handling robotics. We propose to utilize the lectin affinity of Concanavalin A (ConA) coated magnetic beads, which can be applied as a general tool for capturing of *N*-glycosylated peptides and proteins with broad specificity. The isolated glycoproteins will be digested with trypsin and the resulting tryptic peptides purified using ClinProt MB-IMAC Cu beads.

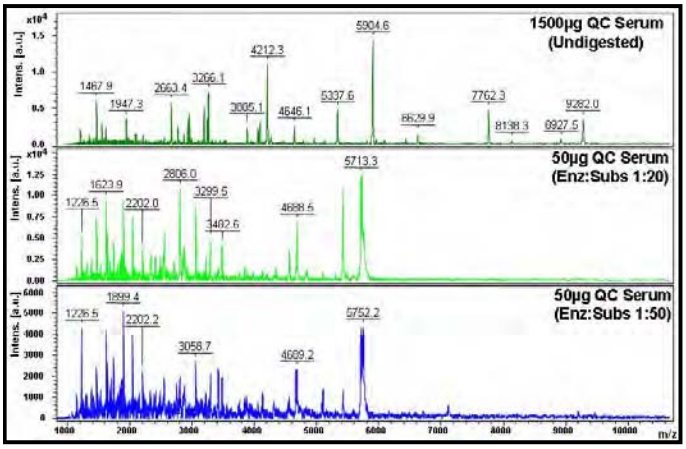

Figure 3. Diluted quality control serum was digested with trypsin at various enzyme to substrate ratios (Enz:Subs). Digested peptides were purified using ClinProt MB-IMAC Cu beads and analyzed by MALDI-TOF MS in linear mode. Undigested neat (undiluted) serum was run directly on ClinProt MB-IMAC Cu as control.

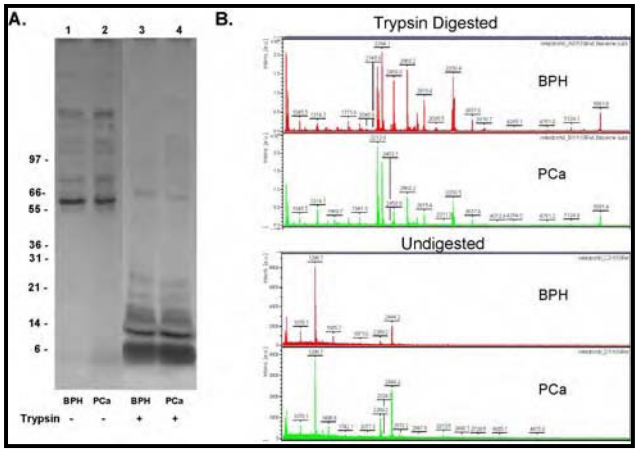

Figure 4. Pooled sera from benign prostatic hyperplasia (BPH) and prostate cancer (PCa) patients were bound to ConA lectin. A portion of bound proteins were separated on 1D SDS-PAGE (A.), either undigested (lanes 1,2) or digested with trypsin (lanes 3,4). (B.) The same eluates, trypsin digested (upper) or undigested (lower), were incubated with MB-IMAC Cu beads and analyzed on Ultraflex™ in linear mode. *Reprinted from* Drake RR et al. *Mol. Cell. Proteomics.* 2006 Jun 7.

Each fraction will be analyzed by MALDI-TOF MS in both the linear and reflector mode. Suitable peptide peaks can then be analyzed by MALDI-TOF/TOF MS to identify the corresponding proteins by database search. For comparison, the same serum will be purified by metal affinity beads (MB-IMAC Cu) without trypsinization. The eluates will be directly analyzed by MALDI-TOF MS (**Figure 3**). Spectra can also be acquired in a much wider mass range in the

linear mode by applying different matrices such as CHCA, SPA, HCCA, 2,5-DHAP to the eluates.

Our approach integrates a high-resolution separation of digest-generated peptides with increasingly sophisticated mass spectrometry for "bottom-up" differential identification. This approach, utilizing primarily fractionation of peptides generated from complex protein mixtures promises a true reflection of the native proteins and that the peptides behave more uniformly in both fractionation and detection. Digest-generated peptides are then subjected to mass spectrometry analysis (**Figure 4**).

3.C. Isolation and identification of the protein biomarkers. We proposed to use the UltrFlex™ TOF/TOF mode for highly sensitive and accurate tandem mass spectrometry for peptide mass fingerprints (PMF). When operated in the TOF/TOF mode the UltraFlex™ achieves very high resolution, accuracy and signal to noise and effective tandem mass spectrometry for protein identification (**Figure 5**).

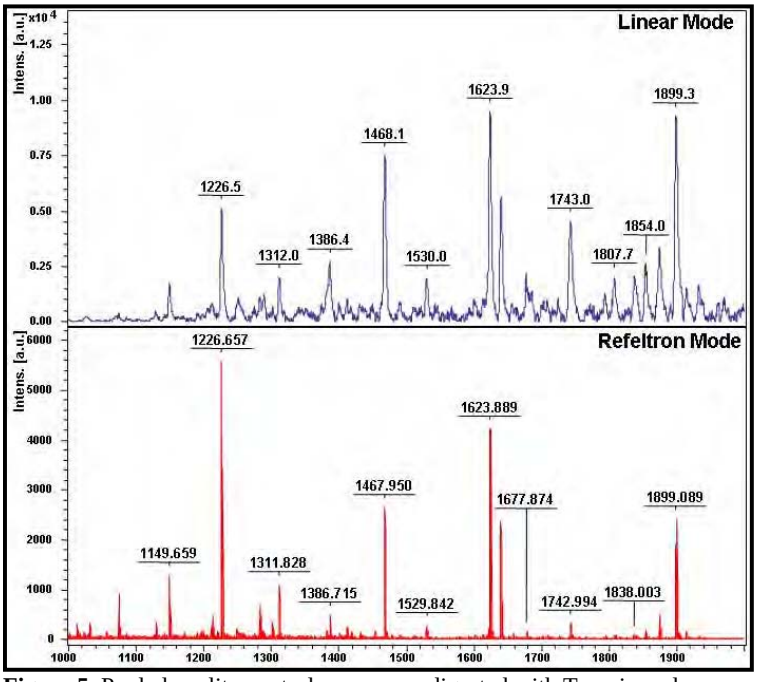

Figure 5. Pooled quality control serum was digested with Trypsin and analyzed by MALDI-TOF MS after purification with ClinProt MB-IMAC Cu beads. The sample fractions were run on both Linear (upper) and Reflector (lower) mode.

Initial analysis of sample fractions in the linear mode will allow for visualization of the target peak. When the peak has been targeted, identification is achieved with the UltraFlex™ which employs ion potential lift (LIFT) in a MALDI-TOF/TOF platform for highly sensitive (attomolar range) and accurate tandem mass spectrometry for peptide mass fingerprints (PMF). Depending on the size of the target peak we will use trypsinized fractions in combination with Laser Induced Dissociation

(LID) for most protein identification and Collision Induced Dissociation (CID) for de novo sequencing and resolving Leu/Ile ambiguity. On the MALDI platform these can be performed serially on the same sample (*see* Suckau et al. 2003 for details).

A major advantage to the combined ClinProt UltraFlex™ system is the ability to directly scale up for isolation and purification prior to applying sequence identification efforts. Our front-end fractionation strategies will help scale up the amount and purity of the sample to facilitate success in the tandem mass operation.

3.D. Development of MS-assisted immunoassays for PCa diagnostics. The ability of the identified and validated biomarkers to diagnose prostate cancer, especially in sample groups where PSA fails to detect cancer (clinical gray area), would be tested using large sample sets on MALDI and SELDI-based immunoassays.

4. REPORTABLE OUTCOMES

4.A. Serum samples from 223 patients with negative prostate biopsy and 185 patients with positive prostate biopsy in the clinical gray area of diagnosis (PSA<4.0ng/ml; Abnormal DRE and/or elevated PSA etc.) are collected and stored in our serum repository for use in this study.

4.B. Initial evaluation of the pre-fractionation of serum samples prior to MALDI-TOF MS has been performed. Based on the results of the initial pilot-experiments, bead-based lectin capture prior to MS analysis generated the best outcome and will be performed in this study. Results of this research are in press (Drake et al. *Mol. Cell. Proteomics* June 2006). Manuscript is attached at the end of the report.

4.C. For protein identification, analysis of tryptic peptides generated from serum were analyzed my MALDI in the TOF/TOF mode. A review of various MS techniques for prostate cancer diagnostics was reported this year in Semmes et al. *J. Cell. Biochem.* June 2006, in which I am a co-author. Manuscript is attached at the end of the report.

4.D. During my postdoctoral training here at EVMS, I was offered a position at the Cancer Therapy and Research Center (CTRC) Institute for Drug Development

(IDD) in San Antonio, Texas. I'll be joining there as a Senior Research Associate from Oct 2, 2006.

5. REFERENCES

1. Adam, B.L., Qu, Y., Davis, J.W., Ward, M.D., Clements, M.A., Cazares, L.H., Semmes, O.J., Schellhammer, P.F., Yasui, Y., Feng, Z., and Wright, G.L., Jr. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. *Cancer Res.* 2002; 62: 3609-3614.
2. Qu Y., Adam B.L., Yasui Y., Ward M.D., Cazares L.H., Schellhammer P.F., Feng Z., Semmes O.J., and Wright G.L., Jr. Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. *Clin. Chem.* 2002; 48: 1835-1843.
3. Malik G., Ward M.D., Gupta S.K., Trosset M.W., Grizzle W.E., Adam B.L., Diaz J.I., and Semmes O.J. (2004). Serum levels of an Isoform of Apolipoprotein A-II as a potential marker for prostate cancer. *Clinical Cancer Research* 2005 Feb 1; 11(3): 1073-1085.
4. Suckau D., Resemann A., Schuerenberg M., Hufnagel P., Franzen J., and Holle A. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Anal. Bioanal. Chem.* 2003; 376: 952-965.
5. Semmes OJ, Malik G, Ward M. Application of mass spectrometry to the discovery of biomarkers for detection of prostate cancer. *J. Cell. Biochem.* 2006 Jun 1; 98(3): 496-503.
6. Dube DH, Bertozzi CR. Glycans in cancer and inflammation--potential for therapeutics and diagnostics. *Nat. Rev. Drug Discov.* 2005 Jun; 4(6): 477-88.
7. Sparbier K, Koch S, Kessler I, Wenzel T, Kostrzewa M. Selective isolation of glycoproteins and glycopeptides for MALDI-TOF MS detection supported by magnetic particles. *J. Biomol. Tech.* 2005 Dec;16(4): 407-13

8. Sparbier K, Wenzel T, Kostrzewa M. Exploring the binding profiles of ConA, boronic acid and WGA by MALDI-TOF/TOF MS and magnetic particles. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2006 Aug 7; 840(1): 29-36.
9. Drake RR, Schwegler EE, Malik G, Diaz JI, Block T, Mehta A, Semmes OJ. Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers *Mol. Cell. Proteomics.* 2006 Jun 7. *In Press*

6. APPENDICES-

APPENDIX I

Reprint:

Authors- Semmes OJ, **Malik G**, Ward M.

Title- Application of mass spectrometry to the discovery of biomarkers for detection of prostate cancer.

Journal- *J. Cell. Biochem.* 2006 Jun 1; 98(3): 496-503.

APPENDIX II

Manuscript:

Authors- Drake RR, Schwegler EE, **Malik G**, Diaz JI, Block T, Mehta A, Semmes OJ.

Title- Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers

Journal- *Mol. Cell. Proteomics.* 2006 Jun 7. *In Press*

Journal of Cellular Biochemistry 98:496–503 (2006)

Application of Mass Spectrometry to the Discovery of Biomarkers for Detection of Prostate Cancer

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Department of Microbiology and Molecular Cell Biology, Center for Biomedical Proteomics, Virginia Prostate Center, Eastern Virginia Medical School, Norfolk Virginia

Abstract There has been an impressive emergence of mass spectrometry based technologies applied toward the study of proteins. Equally notable is the rapid adaptation of these technologies to biomedical approaches in the realm of clinical proteomics. Concerted efforts toward the elucidation of the proteomes of organ sites or specific disease state are proliferating and from these efforts come the promise of better diagnostics/prognostics and therapeutic intervention. Prostate cancer has been a focus of many such studies with the promise of improved care to patients via biomarkers derived from these proteomic approaches. The newer technologies provide higher analytical capabilities, employ automated liquid handling systems, fractionation techniques and bioinformatics tools for greater sensitivity and resolving power, more robust and higher throughput sample processing, and greater confidence in analytical results. In this prospects, we summarize the proteomic technologies applied to date in prostate cancer, along with their respective advantages and disadvantages. The development of newer proteomic strategies for use in future applications is also discussed. J. Cell. Biochem. 98: 496–503, 2006.

Key words: proteomics; prostate cancer; profiling; proteins; peptides; biomarkers

Prostate cancer is the most common non-skin cancer in the US. In 2005, more than 200,000 men have been diagnosed with prostate cancer, and over 30,000 men will die from it, making it the second-most leading cause of cancer-related deaths among men in the US. If diagnosed early, prostate cancer can be effectively treated by surgery or radiation. However, every year, 70,000 men require additional treatment due to recurrence of the disease. Prostate cancer is a complex heterogeneous disease that acts differently in different men. The slow rate of prostate cancer growth, coupled with the widely varied presentation, has made it difficult, if not impossible, to determine conclusively which treatment is best for which man.

Early prostate cancer usually has no symptoms and is most commonly detected through prostate cancer screening tests such as the prostate specific antigen (PSA) blood test and digital rectal exam. An elevated PSA level in the bloodstream does not necessarily indicate prostate cancer, since PSA levels can be altered by infection or other prostate conditions such as benign prostatic hyperplasia (BPH). Although the standard PSA test remains the most widely used screening assay for prostate cancer, approximately 25% of men with prostate cancer have a PSA level below 4.0 ng/ml and only 25% of men with a PSA level of 4–10 ng/ml have prostate cancer. Indeed significant numbers of men with an elevated PSA do not have prostate cancer. Thus there is a need for more accurate and non-invasive techniques to detect, diagnose, and stratify the disease based on molecular markers present in the body fluids.

WHY PROTEOMICS?

Most of the physiological changes in cancer are mediated by molecular alterations at the protein level many of which would not be expected to be revealed at the DNA/RNA level.

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Disease specific changes arising from the tumor cell or microenvironment can be utilized to provide biomarkers that can guide treatment decisions at the molecular level. These biomarker proteins can be uncovered by comprehensive protein analysis of cells, tissues, and body fluids (like blood, seminal plasma, and prostatic fluid) as well as artificially generated animal models and cell lines. Blood has been a particularly attractive target proteome source because cellular biomarkers routinely "leak" into the body fluids. In addition, blood is easy to handle, and acquisition is non-invasive and this proteome likely harbors a true picture of the physiological state of the patient.

Potential proteomic biomarkers of prostate cancer can not only benefit in earliest detection of disease but can also be used for determining cancer risk, stratifying disease stage and grade, monitoring response to therapy, and in general assisting in therapeutic decision making. Through careful sample selection, proper study design, automation in sample handling and processing, proteomic platforms are fast becoming very powerful tools in prostate cancer research.

APPROACHES TO CLINICAL PROTEOMICS

Proteomic studies dating from the 1970s utilized the technique of two-dimensional gel electrophoresis to display a large number of proteins from a given cell-line or organism [O'Farrell, 1975]. The technique works as a powerful tool for comparative analyses of protein expression levels between samples. However it soon became clear that this approach was limited in application with respect to the needs of clinical proteomics. The resolving power is limited by mass and pI, the technique is not high-throughput, the platform is limited in reproducing similar 2-D patterns and needs larger amounts of samples for processing. In response to these limitations researchers have incorporated fluorescent dyes in a process termed 2-D differential in-gel electrophoresis or DIGE [Unlu et al., 1997], to differentially label proteins from multiple sources and analyze the patterns of each on the same 2-D gel. The technique allows for increased throughput and easier comparative expression analysis between samples. Although, these recent advances in staining techniques using fluorescent dyes, along with the use of pre-fractionation approaches [Van den Bergh et al., 2003] and narrower pH ranges in the first dimension along with large format gels [Gorg et al., 2002] are improving the sensitivity and effectiveness (reviewed by [Lilley and Friedman, 2004]), nevertheless a need remains for high-throughput applications capable of simultaneously assessing the proteome of population-representative sample sets.

Combinatorial approaches include a combination of pre-fractionation and gel electrophoresis with mass spectrometry techniques. The approach has been utilized for proteomic analysis of human prostate cancer [Nelson et al., 2000; Ahram et al., 2002; Meehan et al., 2002]. Using a combination of laser capture microdissection, 2-D PAGE followed by LC MS/MS analysis of the tryptic digests of the protein spots, Ahram et al. [2002] identified 40 tumor specific protein expression changes. With the combination of 2-D PAGE, MALDI-TOF MS, peptide mass fingerprinting (PMF) and N-terminal protein sequencing, Meehan et al. [2002] identified 20 protein alterations in prostate tissues and validated them by Western blotting and immuno-histochemistry (IHC). Similarly, the combination of cDNA microarrays, 2-DE and MS has been employed to generate global gene/protein expression profiles of androgen-stimulated prostate cell lines by Nelson et al. [2000].

Even more recently the numbers of technical approaches available to proteomic analysis are proliferating at a staggering pace; to the extent that evaluating the merit of individual approaches has become a top priority as evidenced by several recent requests for applications issued by the National Cancer Institutes. Among the many technical hurdles to successful proteome mining, the two most prominent are likely the daunting numbers of different protein entities and the existing range of protein concentration. Estimates of potential protein types can reach into the millions when considering post-translational modification events and the relative concentration range can span 12 orders of magnitude. Thus, researchers must contend with achieving utility in both resolution and sensitivity of a given technique. An additional level of complexity exists when one considers the heterogeneity of individuals, which is a significant confounding factor in the study design of successful biomarker efforts. Solutions to these issues have come in the form of so called "top down" proteomics in which whole

proteins are separated and case versus control differential established prior to mass spectrometry analysis. Alternatively, complex protein mixtures can be enzymatically digested prior to separation and differential expression determination using mass spectrometry in "bottom up" approaches. This latter approach was enabled via improved tandem mass spectrometry advances. In all of these conceptual approaches (see Fig. 1), "front-end" sample fractionation and separation strategies are required to reduce the complexity of native clinical samples (or cell lysates) and the technical improvements in this area have grown as well.

SCREENING ALTERNATIVES TO MS-BASED PROTEOMICS

There are several very exciting approaches to high-throughput screening proteomics approaches that will not be discussed in detail here. These include antibody arrays (reviewed by [Haab, 2005]) which have had and will continue to have significant applications in cancer research. The antibody-arrays have been utilized for protein profiling, biomarker identification, protein characterization and in some cases the detection of protein post-translational modifications. Some notable success stories of interest to

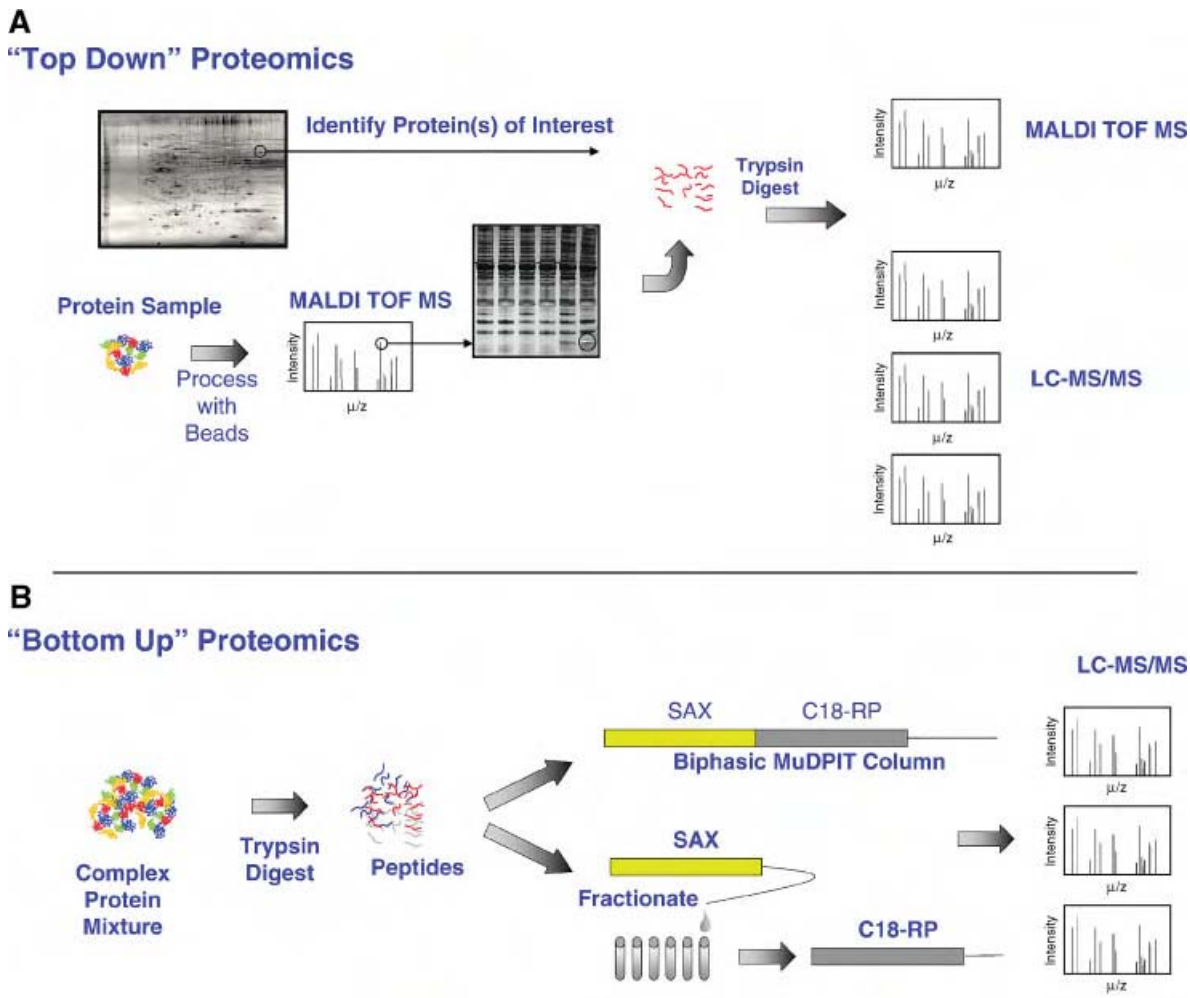

Fig. 1. Two major approaches to clinical proteomics. **A**: In the Top-down proteomics approach, whole proteins are pre-fractionated via various gel and non-gel based techniques. There are obvious scientific advantages for determining protein complexes and post-translational modifications when employing these approaches. Intact proteins of interest are then subjected to subsequent MS-based analyses using either single or tandem mass spectrometry. Throughput is usually indirectly proportional to the amount of information gathered. **B**: The bottom-up proteomics approach utilizes primarily non-gel based fractionation of peptides generated from complex protein mixtures. The rationale is that the digested peptides will reflect the native proteins and that the peptides behave more uniformly in both fractionation and detection. Digest-generated peptides are then subjected to mass spectrometry analysis. The approach can involve simple peptide mass profiling as well as quantitative tandem mass spectrometry to yield protein identification and relative protein concentration.

this audience would be in the analysis of proteins in tumors [Knezevic et al., 2001], the specific analysis of the prostate cancer humoral antibody response in patients to antigens [Webb et al., 1981; Wang et al., 2005a] as well as serum protein expression profiling [Miller et al., 2003]. The development of tissue microarray (TMA) technology [Kononen et al., 1998] has initiated large-scale studies using tumor tissues. The technique has also been widely extended to prostate cancer studies (reviewed by [Kuefer et al., 2004; Watanabe et al., 2005]) specifically for protein expression profiling, biomarker validation [Rubin et al., 2002; Zellweger et al., 2003], and study of tumor biology [Sun et al., 2003; Boddy et al., 2005; Chuan et al., 2005]. TMA technology has considerable value in translating the information gained from initial discovery into clinical applications. Protein arrays have been used to detect antibodies in samples against a set of cancer antigens [Robinson et al., 2002]. This approach may also involve the arraying of uniquely designed antigens and has been successfully applied in prostate cancer studies using patient samples or cancer models [Lagarkova et al., 2003; Nishizuka et al., 2003; Zhang et al., 2003]. Thus, it is essential that the value of these approaches not be lost in the focus of this review on the emerging mass spectrometry based techniques.

MS-BASED APPROACHES TO CLINICAL PROTEOMICS

Top-down: The highly complex and wide dynamic range of proteins/peptides in body fluids needs high-resolution systems for biomarker mining. Moreover, in complex body fluids like serum, the biomarkers could be spread over a wide range of concentrations. One of the ways to ease the "mining" of biomarkers in complex proteomes is via the separation of whole proteins prior to MS-based analyses. Two-dimensional liquid chromatography-based technologies (2D-LC) are the most widely used techniques for the this type of approach [Yan et al., 2003; Kolch et al., 2005; Qin et al., 2005]. Pre-fractionation of samples in liquid phase prior to biomarker mining not only reduces the proteome complexity of body fluids like serum but also allows for automation of sample processing before the analyses of the fractions. Capillary electrophoresis coupled to mass spectrometry has also been utilized in several studies for a high resolution fast separation of complex fluids like urine [Chalmers et al., 2005; Fliser et al., 2005].

Protein expression profiling using either MALDI-TOF or SELDI-TOF approaches has seen a wide application to many disease sites including prostate cancer [Peter et al., 2001; Adam et al., 2002; Cazares et al., 2002; Petricoin et al., 2002; Qu et al., 2002; Banez et al., 2003; Kidd et al., 2003; Lehrer et al., 2003]. The SELDI-TOF approach utilizes a chip-based affinity capture procedure to reduce sample complexity and then "profiles" the bound intact proteins by mass. The technique is sensitive, needs minimal amount of protein, and is relatively high throughput (reviewed by [Wright, 2002; Conrads et al., 2004; Solassol et al., 2005]). Our laboratory and others have been employing a combination of chromatographic paramagnetic beads and MALDI TOF/TOF MS to present a powerful and sensitive analysis of pre-fractionated samples (reviewed by [Pusch and Kostrzewa, 2005]). The paramagnetic beads allow for reasonable high throughput processing and reproducible fractionation of proteins/peptides followed by MALDI-TOF MS analysis (Fig. 2). Since the introduction of this technology to the field, the technique has been widely used for single or multidimensional separation of proteins/peptides on the beads. The fractions are then spotted on target plates for MALDI-TOF analysis [Villanueva et al., 2004]. Although not yet fully realized, this approach via sophisticated TOF/TOF capabilities offers direct protein identification with little or no additional work-up. We have been particularly interested in the utility of this instrumentation in improving so called immuno-MS (Fig. 2) which is an approach that we first reported on in early 2000 using SELDI-TOF. The incorporation of immuno-MS provides for early validation of biomarkers discovered on the same platform and offers distinct advantage over ELISA in that isoforms, modifications and cleavage products can be evaluated with the same antibody.

Bottom up: Various non-gel based liquid chromatography techniques focusing on peptides are gaining attention, as they allow multidimensional, automated separation of peptides representing very low abundance of proteins. The capabilities of these techniques to perform proteome analysis from minimal samples has

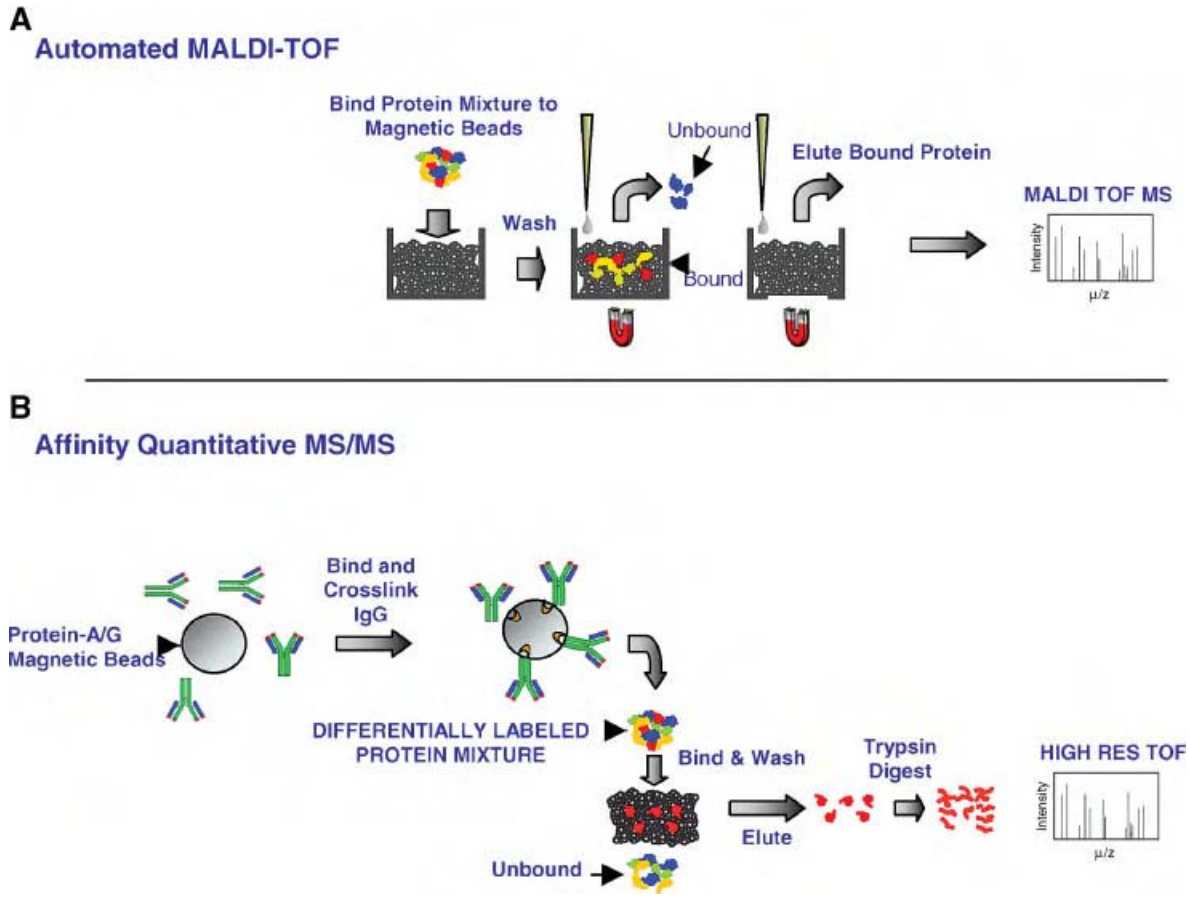

Fig. 2. Introduction of automated high-resolution MALDI-TOF based approaches (**A**) Using a combination of affinity selection on paramagnetic beads and downstream mass spectrometry, a high-resolution and high-throughput MALDI-TOF based approach has been developed. The technique utilizes the affinity-capture of proteins/peptides from complex fluids using a variety of capture molecules. The bound/unbound protein fractions can then be spotted on a target plate and analyzed by MALDI-TOF MS. The procedure profoundly reduces the sample complexity and then ''profiles'' the bound intact proteins by mass. **B**: The automated paramagnetic bead system can also be used in combination with differential protein labeling for a quantitative MS analysis. Using mass-shift and mass-defect tagging of protein mixtures from different sources, the high resolution MALDI-TOF/TOF approach allows for direct protein quantitation and identification.

generated new prospects for biomarker discovery especially using selected cell populations from tissue specimens [Wang et al., 2005b]. A high-resolution chromatographic separation of digest-generated peptides prior to mass spectrometry analysis without the involvement of gel electrophoresis has potential application to clinical proteomics. Such micro fluidic systems have already been integrated with increasingly sophisticated mass spectrometry for bottom-up differential identification [Brivio et al., 2002; Li et al., 2002; Zhu et al., 2003; Metodiev et al., 2004]. Peptide quantification using a combination of multidimensional liquid-chromatography, protein labeling and digestion has also been reported recently [Gygi et al., 1999; Griffin et al., 2003; DeSouza et al., 2005] adding direct quantitation and thus making it a much more powerful tool. The technique of differential peptide display (DPD) has been recently utilized to analyze the peptidome of the HUPO human serum and plasma specimens [Tammen et al., 2005]. The samples were fractionated on RP-HPLC and each fraction is applied to MALDI-TOF MS to generate an in silico 2-D display of peptide masses. A combinatorial approach of protein fractionation using HPLC, tryptic digestion and RPLC-MS/MS has also been recently utilized to characterize the mouse serum proteome [Hood et al., 2005]. Using these techniques the group identified 12,300 unique peptides originating from 4567 unique mouse serum proteins.

A number of groups are now trying to identify and analyze proteins from less complex mixtures such as seminal fluids [Utleg et al., 2003;

Fung et al., 2004], laser captured cells from cancer tissues [Paweletz et al., 2001; Cazares et al., 2002; Diaz et al., 2004], albumin-associated proteins from blood sera [Lowenthal et al., 2005], glycosylated proteins [Manning et al., 2004; Yang and Hancock, 2005; Yang et al., 2005], or sub cellular fractions from cancer cells [Gretzer et al., 2004]. The up-front reduction in sample complexity helps to reduce the numbers of proteins being interrogated thus effectively increasing the coverage of the disease proteome.

Clearly there is a demand for enabling the adaptation of cutting-edge mass spectroscopy approaches to clinical proteomics. These solutions will likely focus on improving sample acquisition and handling, reducing sample complexity, increasing sample throughput, and improving sensitivity/resolution in ion detection. It is also clear that this need, delineated by the pioneering work of a handful of clinical proteomics laboratories, has been noted by the mainstream mass spectrometry community. However, technology alone cannot drive future success in the application of proteomics to prostate cancer. Clearly, a concerted multi-disciplinary effort is needed. Central to this collaboration is the biochemist with a greater understanding of protein behavior and the tools to tease proteins from the proteome. In fact the advances in the application of mass spectrometry to proteins should signal a renaissance in classic biochemistry, an expertise that had largely given way to molecular biology. Studies involving mouse models, cell lines and direct human samples need to be coordinated toward the same clinical goals. For example, uncovering the proteome changes associated with exposure of LNCaP cells to androgen [Meehan and Sadar, 2004], would compliment nicely with similar studies in androgen resistant mouse models and proteomic analysis of prostatic fluids from patients with androgen-resistance transition. In the end, study design and solid biochemistry will push the success envelope of new technologies in clinical proteomics.

REFERENCES

Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, Semmes OJ, Schellhammer PF, Yasui Y, Feng Z, Wright GL, Jr. 2002. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res 62:3609–3614.

Ahram M, Best CJ, Flaig MJ, Gillespie JW, Leiva IM, Chuaqui RF, Zhou G, Shu H, Duray PH, Linehan WM, Raffeld M, Ornstein DK, Zhao Y, Petricoin EF III, Emmert-Buck MR. 2002. Proteomic analysis of human prostate cancer. Mol Carcinog 33:9–15.

Banez LL, Prasanna P, Sun L, Ali A, Zou Z, Adam BL, McLeod DG, Moul JW, Srivastava S. 2003. Diagnostic potential of serum proteomic patterns in prostate cancer. J Urol 170:442–446.

Boddy JL, Fox SB, Han C, Campo L, Turley H, Kanga S, Malone PR, Harris AL. 2005. The androgen receptor is significantly associated with vascular endothelial growth factor and hypoxia sensing via hypoxia-inducible factors HIF-1a, HIF-2a, and the prolyl hydroxylases in human prostate cancer. Clin Cancer Res 11:7658–7663.

Brivio M, Fokkens RH, Verboom W, Reinhoudt DN, Tas NR, Goedbloed M, van den Berg A. 2002. Integrated microfluidic system enabling (bio)chemical reactions with on-line MALDI-TOF mass spectrometry. Anal Chem 74:3972–3976.

Cazares LH, Adam BL, Ward MD, Nasim S, Schellhammer PF, Semmes OJ, Wright GL, Jr. 2002. Normal, benign, preneoplastic, and malignant prostate cells have distinct protein expression profiles resolved by surface enhanced laser desorption/ionization mass spectrometry. Clin Cancer Res 8:2541–2552.

Chalmers MJ, Mackay CL, Hendrickson CL, Wittke S, Walden M, Mischak H, Fliser D, Just I, Marshall AG. 2005. Combined top-down and bottom-up mass spectrometric approach to characterization of biomarkers for renal disease. Anal Chem 77:7163–7171.

Chuan Y, Pang ST, Bergh A, Norstedt G, Pousette A. 2005. Androgens induce CD-9 in human prostate tissue. Int J Androl 28:291–296.

Conrads TP, Hood BL, Issaq HJ, Veenstra TD. 2004. Proteomic patterns as a diagnostic tool for early-stage cancer: A review of its progress to a clinically relevant tool. Mol Diagn 8:77–85.

DeSouza L, Diehl G, Rodrigues MJ, Guo J, Romaschin AD, Colgan TJ, Siu KW. 2005. Search for cancer markers from endometrial tissues using differentially labeled tags iTRAQ and cICAT with multidimensional liquid chromatography and tandem mass spectrometry. J Proteome Res 4:377–386.

Diaz JI, Cazares LH, Corica A, John Semmes O. 2004. Selective capture of prostatic basal cells and secretory epithelial cells for proteomic and genomic analysis. Urol Oncol 22:329–336.

Fliser D, Wittke S, Mischak H. 2005. Capillary electrophoresis coupled to mass spectrometry for clinical diagnostic purposes. Electrophoresis 26:2708–2716.

Fung KY, Glode LM, Green S, Duncan MW. 2004. A comprehensive characterization of the peptide and protein constituents of human seminal fluid. Prostate 61:171–181.

Gorg A, Boguth G, Kopf A, Reil G, Parlar H, Weiss W. 2002. Sample prefractionation with Sephadex isoelectric focusing prior to narrow pH range two-dimensional gels. Proteomics 2:1652–1657.

Gretzer MB, Chan DW, van Rootselaar CL, Rosenzweig JM, Dalrymple S, Mangold LA, Partin AW, Veltri RW.

2004. Proteomic analysis of dunning prostate cancer cell lines with variable metastatic potential using SELDI-TOF. Prostate 60:325–331.

Griffin TJ, Lock CM, Li XJ, Patel A, Chervetsova I, Lee H, Wright ME, Ranish JA, Chen SS, Aebersold R. 2003. Abundance ratio-dependent proteomic analysis by mass spectrometry. Anal Chem 75:867–874.

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17:994–999.

Haab BB. 2005. Antibody arrays in cancer research. Mol Cell Proteomics 4:377–383.

Hood BL, Zhou M, Chan KC, Lucas DA, Kim GJ, Issaq HJ, Veenstra TD, Conrads TP. 2005. Investigation of the mouse serum proteome. J Proteome Res 4:1561–1568.

Kidd LC, Woodson K, Taylor PR, Albanes D, Virtamo J, Tangrea JA. 2003. Polymorphisms in glutathione-S-transferase genes (GST-M1, GST-T1 and GST-P1) and susceptibility to prostate cancer among male smokers of the ATBC cancer prevention study. Eur J Cancer Prev 12:317–320.

Knezevic V, Leethanakul C, Bichsel VE, Worth JM, Prabhu VV, Gutkind JS, Liotta LA, Munson PJ, Petricoin EF III, Krizman DB. 2001. Proteomic profiling of the cancer microenvironment by antibody arrays. Proteomics 1:1271–1278.

Kolch W, Neususs C, Pelzing M, Mischak H. 2005. Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 24:959–977.

Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. 1998. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4:844–847.

Kuefer R, Hofer MD, Gschwend JE, Rubin MA. 2004. Tissue microarrays. High-throughput procedures to verify potential biomarkers. Urologe A 43:659–667.

Lagarkova MA, Koroleva EP, Kuprash DV, Boitchenko VE, Kashkarova UA, Nedospasov SA, Shebzukhov YV. 2003. Evaluation of humoral response to tumor antigens using recombinant expression-based serological mini-arrays (SMARTA). Immunol Lett 85:71–74.

Lehrer S, Roboz J, Ding H, Zhao S, Diamond EJ, Holland JF, Stone NN, Droller MJ, Stock RG. 2003. Putative protein markers in the sera of men with prostatic neoplasms. BJU Int 92:223–225.

Li J, LeRiche T, Tremblay TL, Wang C, Bonneil E, Harrison DJ, Thibault P. 2002. Application of microfluidic devices to proteomics research: Identification of trace-level protein digests and affinity capture of target peptides. Mol Cell Proteomics 1:157–168.

Lilley KS, Friedman DB. 2004. All about DIGE: Quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics 1:401–409.

Lowenthal MS, Mehta AI, Frogale K, Bandle RW, Araujo RP, Hood BL, Veenstra TD, Conrads TP, Goldsmith P, Fishman D, Petricoin EF III, Liotta LA. 2005. Analysis of albumin-associated peptides and proteins from ovarian cancer patients. Clin Chem 51:1933–1945.

Manning JC, Seyrek K, Kaltner H, Andre S, Sinowatz F, Gabius HJ. 2004. Glycomic profiling of developmental changes in bovine testis by lectin histochemistry and further analysis of the most prominent alteration on the level of the glycoproteome by lectin blotting and lectin affinity chromatography. Histol Histopathol 19:1043–1060.

Meehan KL, Sadar MD. 2004. Quantitative profiling of LNCaP prostate cancer cells using isotope-coded affinity tags and mass spectrometry. Proteomics 4:1116–1134.

Meehan KL, Holland JW, Dawkins HJ. 2002. Proteomic analysis of normal and malignant prostate tissue to identify novel proteins lost in cancer. Prostate 50:54–63.

Metodiev MV, Timanova A, Stone DE. 2004. Differential phosphoproteome profiling by affinity capture and tandem matrix-assisted laser desorption/ionization mass spectrometry. Proteomics 4:1433–1438.

Miller JC, Zhou H, Kwekel J, Cavallo R, Burke J, Butler EB, Teh BS, Haab BB. 2003. Antibody microarray profiling of human prostate cancer sera: Antibody screening and identification of potential biomarkers. Proteomics 3:56–63.

Nelson PS, Han D, Rochon Y, Corthals GL, Lin B, Monson A, Nguyen V, Franza BR, Plymate SR, Aebersold R, Hood L. 2000. Comprehensive analyses of prostate gene expression: Convergence of expressed sequence tag databases, transcript profiling and proteomics. Electrophoresis 21:1823–1831.

Nishizuka S, Charboneau L, Young L, Major S, Reinhold WC, Waltham M, Kouros-Mehr H, Bussey KJ, Lee JK, Espina V, Munson PJ, Petricoin E III, Liotta LA, Weinstein JN. 2003. Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. Proc Natl Acad Sci USA 100:14229–14234.

O'Farrell PH. 1975. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021.

Paweletz CP, Liotta LA, Petricoin EF III. 2001. New technologies for biomarker analysis of prostate cancer progression: Laser capture microdissection and tissue proteomics. Urology 57:160–163.

Peter J, Unverzagt C, Krogh TN, Vorm O, Hoesel W. 2001. Identification of precursor forms of free prostate-specific antigen in serum of prostate cancer patients by immunosorption and mass spectrometry. Cancer Res 61:957–962.

Petricoin EF III, Ornstein DK, Paweletz CP, Ardekani A, Hackett PS, Hitt BA, Velassco A, Trucco C, Wiegand L, Wood K, Simone CB, Levine PJ, Linehan WM, Emmert-Buck MR, Steinberg SM, Kohn EC, Liotta LA. 2002. Serum proteomic patterns for detection of prostate cancer. J Natl Cancer Inst 94:1576–1578.

Pusch W, Kostrzewa M. 2005. Application of MALDI-TOF mass spectrometry in screening and diagnostic research. Curr Pharm Des 11:2577–2591.

Qin S, Ferdinand AS, Richie JP, O'Leary MP, Mok SC, Liu BC. 2005. Chromatofocusing fractionation and two-dimensional difference gel electrophoresis for low abundance serum proteins. Proteomics 5:3183–3192.

Qu Y, Adam BL, Yasui Y, Ward MD, Cazares LH, Schellhammer PF, Feng Z, Semmes OJ, Wright GL, Jr. 2002. Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. Clin Chem 48:1835–1843.

Robinson WH, DiGennaro C, Hueber W, Haab BB, Kamachi M, Dean EJ, Fournel S, Fong D, Genovese MC, de Vegvar HE, Skriner K, Hirschberg DL, Morris RI,

Muller S, Pruijn GJ, van Venrooij WJ, Smolen JS, Brown PO, Steinman L, Utz PJ. 2002. Autoantigen microarrays for multiplex characterization of autoantibody responses. Nat Med 8:295–301.

Rubin MA, Dunn R, Strawderman M, Pienta KJ. 2002. Tissue microarray sampling strategy for prostate cancer biomarker analysis. Am J Surg Pathol 26:312–319.

Solassol J, Marin P, Maudelonde T, Mange A. 2005. Proteomic profiling: The potential of Seldi-Tof for the identification of new cancer biomarkers. Bull Cancer 92: 763–768.

Sun YX, Wang J, Shelburne CE, Lopatin DE, Chinnaiyan AM, Rubin MA, Pienta KJ, Taichman RS. 2003. Expression of CXCR4 and CXCL12 (SDF-1) in human prostate cancers (PCa) in vivo. J Cell Biochem 89:462–473.

Tammen H, Schulte I, Hess R, Menzel C, Kellmann M, Mohring T, Schulz-Knappe P. 2005. Peptidomic analysis of human blood specimens: Comparison between plasma specimens and serum by differential peptide display. Proteomics 5:3414–3422.

Unlu M, Morgan ME, Minden JS. 1997. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. Electrophoresis 18:2071–2077.

Utleg AG, Yi EC, Xie T, Shannon P, White JT, Goodlett DR, Hood L, Lin B. 2003. Proteomic analysis of human prostasomes. Prostate 56:150–161.

Van den Bergh G, Clerens S, Vandesande F, Arckens L. 2003. Reversed-phase high-performance liquid chromatography prefractionation prior to two-dimensional difference gel electrophoresis and mass spectrometry identifies new differentially expressed proteins between striate cortex of kitten and adult cat. Electrophoresis 24: 1471–1481.

Villanueva J, Philip J, Entenberg D, Chaparro CA, Tanwar MK, Holland EC, Tempst P. 2004. Serum peptide profiling by magnetic particle-assisted, automated sample processing and MALDI-TOF mass spectrometry. Anal Chem 76:1560–1570.

Wang X, Yu J, Sreekumar A, Varambally S, Shen R, Giacherio D, Mehra R, Montie JE, Pienta KJ, Sanda MG, Kantoff PW, Rubin MA, Wei JT, Ghosh D, Chinnaiyan AM. 2005a. Autoantibody signatures in prostate cancer. N Engl J Med 353:1224–1235.

Wang Y, Balgley BM, Lee CS. 2005b. Tissue proteomics using capillary isoelectric focusing-based multidimensional separations. Expert Rev Proteomics 2:659–667.

Watanabe A, Cornelison R, Hostetter G. 2005. Tissue microarrays: Applications in genomic research. Expert Rev Mol Diagn 5:171–181.

Webb KS, Ware JL, Parks SF, Paulson DF. 1981. A serologic approach to the definition of human prostatic carcinoma antigens. Prostate 2:369–380.

Wright GL, Jr. 2002. SELDI proteinchip MS: A platform for biomarker discovery and cancer diagnosis. Expert Rev Mol Diagn 2:549–563.

Yan F, Sreekumar A, Laxman B, Chinnaiyan AM, Lubman DM, Barder TJ. 2003. Protein microarrays using liquid phase fractionation of cell lysates. Proteomics 3:1228–1235.

Yang Z, Hancock WS. 2005. Monitoring glycosylation pattern changes of glycoproteins using multi-lectin affinity chromatography. J Chromatogr A 1070:57–64.

Yang Z, Hancock WS, Chew TR, Bonilla L. 2005. A study of glycoproteins in human serum and plasma reference standards (HUPO) using multilectin affinity chromatography coupled with RPLC-MS/MS. Proteomics 5:3353–3366.

Zellweger T, Ninck C, Mirlacher M, Annefeld M, Glass AG, Gasser TC, Mihatsch MJ, Gelmann EP, Bubendorf L. 2003. Tissue microarray analysis reveals prognostic significance of syndecan-1 expression in prostate cancer. Prostate 55:20–29.

Zhang JY, Casiano CA, Peng XX, Koziol JA, Chan EK, Tan EM. 2003. Enhancement of antibody detection in cancer using panel of recombinant tumor-associated antigens. Cancer Epidemiol Biomarkers Prev 12:136–143.

Zhu K, Kim J, Yoo C, Miller FR, Lubman DM. 2003. High sequence coverage of proteins isolated from liquid separations of breast cancer cells using capillary electrophoresis-time-of-flight MS and MALDI-TOF MS mapping. Anal Chem 75:6209–6217.

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Lectin Capture Strategies Combined with Mass Spectrometry for the Discovery of Serum Glycoprotein Biomarkers

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ABSTRACT

The application of mass spectrometry to identify disease biomarkers in clinical fluids like serum using high-throughput protein expression profiling continues to evolve as technology development, clinical study design, and bioinformatics improve. Previous protein expression profiling studies have offered needed insight into issues of technical reproducibility, instrument calibration, sample preparation, study design and supervised bioinformatics data analysis. In this overview, new strategies to increase the utility of protein expression profiling for clinical biomarker assay development are discussed, with an emphasis on utilizing differential lectin-based glycoprotein capture and targeted immuno-based assays. The carbohydrate binding specificities of different lectins offers a biological affinity approach that complements existing mass spectrometer capabilities and retains automated throughput options. Specific examples using serum samples from prostate cancer and hepatocellular carcinoma subjects are provided, along with suggested experimental strategies for integration of lectin based methods into clinical fluid expression profiling strategies. Our example workflow incorporates the necessity of early validation in biomarker discovery using an immuno-based targeted analytical approach that integrates well with upstream discovery technologies.

Serum protein expression profiling using time-of-flight mass spectrometry approaches to identify biomarkers of disease has reached a nexus of technology development, clinical study design, and bioinformatics. Following a period of promising initial work using matrix-assisted laser desorption ionization (MALDI) and surface-enhanced laser assisted ionization (SELDI) mass spectrometry (1-4), new strategies to increase the utility of this approach for clinical biomarker assay development are needed (5,6). Largely employing simple chemical affinity beads or surfaces to decrease the sample complexities, these methods offer an automated, sensitive technique that consumes small amounts of clinical sample with relatively high throughput (2, 7-9). Concerns with the approach have included lack of analytical reproducibility, diminished robustness of discovered biomarkers during validation, lack of protein identification, and a fear that the dynamic range of prevalent proteins in serum or plasma prohibits identification of proteins associated with disease (10-12). Some of these concerns have subsequently been attributed to study design bias, chance, an overgeneralization of results and sample processing issues (13,14). On the other hand, when careful study design and sample handling is combined with carefully controlled instrument calibration, automated sample preparation, and supervised bioinformatic data analysis, serum expression profiling can be reproducible and portable across multiple laboratories (5,8, 15-17). However, the remaining issues of protein dynamic range and complexity continue to plague these and indeed all proteomic approaches.

At this point in time, future expression profiling studies using clinical samples will require a careful balance of controlling for known problems while at the same time exploiting the rapid advances in robotic fractionation and mass spectrometry

technologies. In this overview of emerging serum proteomic expression profiling strategies, we propose that use of lectin targeting of serum glycoprotein isoforms provides the desired experimental balance that minimizes known study design concerns while retaining the established strengths of high-throughput approaches. The carbohydrate binding specificities of different lectins offers a biological affinity approach that complements existing chemical affinity methods and retains automated throughput options with current mass spectrometer capabilities. Specific examples using serum samples from prostate cancer and hepatocellular carcinoma subjects are provided, along with suggested experimental strategies for integration of lectin based methods into clinical fluid expression profiling strategies.

Altered Glycoproteins and Cancer

It has long been known that cellular glycosylation profiles change significantly during oncogenesis (18-20), and hence the continued search for tumor-secreted glycoproteins that can serve as biomarkers for diagnostics and/or tumor markers of the biological changes associated with the altered glycosylation patterns associated with development of cancer (21-24). One well characterized example is that of increased activity of N-Acetylglucosaminyltransferase V (GnT-V), a key enzyme in the formation of branching asparagine-linked oligosaccharides that has been linked to tumor invasion and metastasis in multiple cancers (25-28). An increase of ß1-6 branched oligosaccharides within metastatic lymph nodes of breast carcinomas has been reported, and the presence of the branched oligosaccharides was associated with poor prognosis (28). The role of sialylated oligosaccharides was evaluated within primary breast tumors,

and it was found that an overall reduction in the diversity of sialylated and neutral oligosaccharides occurred with disease progression (29, 30). It is also clear that fucosylated glycoproteins are elevated in individuals with liver, colorectal and prostate cancers (18, 31). As discussed in a later section, we have also shown that a comprehensive characterization of differentially fucosylated serum glycoproteins can be used to readily distinguish hepatitis B induced liver cancer subjects from healthy control subjects in blinded assays (32, 33).

Prostate specific antigen (PSA) is one of the best characterized examples of a secreted glycoprotein used in cancer diagnostics, and multiple glycoforms of PSA have been described (31, 34-36). PSA is a 28,400 Da glycoprotein with one defined N-linked oligosaccharide side chain at Asn45, and is a serine protease in the kallikrein family (kallikrein 3) (37). PSA is secreted primarily by prostatic epithelial cells into the seminal plasma, where it can reach concentrations of 0.5–3 mg/ml (38). The glycoforms of PSA from seminal plasma have been shown to differ from the glycosylation of PSA secreted by the prostate metastatic tumor cell line LNCaP (31, 34, 35). A thorough comparison of PSA glycoforms from seminal plasma and serum from healthy control and prostate cancer patients has been described (36), however, larger scale studies with more clinical emphasis on study design and sample numbers are still needed. Additionally, evaluation of any differences in the glycoforms of PSA bound by carrier proteins in serum (primarily α-1-antichymotrypsin) versus that of the free circulating PSA remains to be determined. Alpha-fetoprotein (AFP) is another well characterized serum glycoprotein used as a surrogate marker of the presence of liver cancers, and multiple glycoforms of AFP have been identified (33, 39-41). Specific targeting of these PSA and AFP

glycoforms as the diagnosis for disease state, or similar characterization of other known serum components like prostate specific membrane antigen (PSMA), represent a targeted proteomic strategy amenable to quantitative mass spectrometry strategies that employ isotope-tagging or mass-shift labeling techniques (42, 43).

Glycoproteomics and Lectins

The term glycoproteomics has been used to describe this emerging branch of proteomics that focuses on characterizing the protein and carbohydrate constituents of glycoproteins. Structural elucidation of mammalian glycoproteins has long relied upon the use of lectins, a class of proteins found in plants, bacteria, fungi and animals that are known to bind specific oligosaccharide moieties (44-47). Unlike antigen-antibody binding affinities, the affinity constants (K_a) for the binding of monosaccharides and oligosaccharides to most lectins are in the low micromolar range, but can be millimolar (47,48). For affinity capture purposes, it is the multivalent nature of both the oligosaccharides and the lectins themselves that make these interactions useful for chromatography separations (46,47). The most common approaches for use of lectins to capture serum glycoproteins has been to digest the serum with trypsin, isolate the glycopeptides with one or more lectins linked to a support resin, elute and deglycosylate the bound peptides with Protein N-Glycanase F (PNGaseF). The sequence and protein identities of these peptides are determined by tandem mass spectrometry (42, 49-53) or Fourier transform ion-cyclotron resonance (FT-ICR) mass spectrometry (54-56). These approaches have been proven to be useful for identifying low concentration serum glycoproteins, but there is very low sample throughput. Strategies that probe different

lectins bound on multiple array platforms (57-59) are emerging as one approach to overcome the throughput issue. These assays, along with different nanotechnology improvements (60,61), will continue to evolve toward potential clinical assays.

In this overview, we will describe our approach to the application of different lectins to enrich for serum glycoforms found in sera from prostate cancer and hepatocellular carcinoma subjects. For the lectin affinity approach, we believe this offers several distinct biochemical advantages toward protein fractionation. In addition, there are many lectin types available commercially in pure, resin-bound, biotinylated or fluorescent-labeled forms, and they are generally inexpensive. These properties lend themselves to automation, in particular bead-based automated fractionation strategies as front-ends to mass spectrometry (62). Many major serum proteins, in particular albumin, are not glycosylated and are therefore not bound efficiently by lectins, offering a concomitant decrease in dynamic range of serum protein targets. Therefore, the biological affinity of lectins offers multiple uses and strategies to maximize the information gained from precious clinical samples. Issues related to uniformity in preparation of different lectins, their known weak binding constants and reproducibility of binding across large sample numbers are clear hurdles that will need to be addressed as higher throughput automation strategies are pursued. In the following paragraphs, some of these properties are demonstrated for isolation of serum glycoproteins, and strategies to automate these processes while maximizing the amount of information gathered from the analysis will be discussed.

Fractionation and Identification of Lectin Captured Serum Glycoproteins

In order to capitalize on the many glycoproteins found in serum and the known changes in glycosylation associated with cancers we have devised a modular strategy for specific targeting of glycoproteins in sera for characterization as potential biomarkers. As shown in the schematic in figure 1, the up-front differential lectin affinity capture module integrates well with a variety of proteomic tools and resources currently available to enrich, identify and characterize serum proteins. As described, our approach of stratifying whole glycoproteins is in contrast to the more typical paradigm of isolating glycopeptides prior to tandem mass spectrometry identification (Figure 1, left branch), we are attempting to retain the larger multi-protein complexes in serum via lectin capture prior to trypsin digestion and tandem mass spectrometry (Figure 1, right branch). Thus, the information gained will be complimentary to a glycopeptide-based analysis. The modularity of the lectin affinity step is that it is not dependent on any one method or mass spectrometry platform, and is inherently extensible and adaptable to new technology improvements at any point in the process. It is also amenable to automation with bead-based or chip-based robotics, which is important in large scale assessment of sera from well defined clinical sets.

Initially we assessed two parameters, which lectins provided the most differential capture, and whether serum depletion prior to lectin capture improved detection of glycoproteins. A panel of six lectins was employed in the capture step. These included two lectins known to bind fucose residues linked to N-acetylglucosamine, AAL (*Aleuria aurantia*) and AAA, (*Anguilla anguilla*), a lectin targeting a2,6 linked sialic acid residues, SNA1 (*Sambucus nigra*), and HPA (*Helix pomatia* agglutinin) which binds N-acetylgalactosamine residues. Two broad coverage lectins, wheat germ agglutinin (WGA,

binds terminal N-acetylglucosamines) and Concanavalin A (ConA, binds terminal mannoses and glucoses) were also included. The lectin incubations were initially done with pooled sera from matched subjects with benign hyperplasia (BPH) and prostate cancer (PCa) (n = 5). Sera were incubated with each lectin bound to agarose (E-Y Laboratories or Vector Laboratories) for 16 hrs, eluted with the appropriate target monsaccharide and separated by SDS-PAGE as we previously described (32,63). Representative separations of the eluted proteins from each lectin are shown in figure 2. It is clear from this simple gel-based analysis that use of different lectins confers selective enrichment of serum glycoproteins, and some overlap and redundancy in the captured proteins. Each of these findings is consistent with known biology of glycoproteins.

Gel slices of multiple protein bands from SDS polyacrylamide gels, like that shown in figure 2, were prepared for analysis by LC-MS/MS or MALDI-TOF/TOF. A partial list of the proteins identified by this approach that are differentially expressed between BPH and PCa sera are presented in table 1, listed per lectin. Not surprisingly, these are primarily proteins found in high concentrations in serum, so that it is specifically the variant in glycosylation that is associated with disease. Such results of course require orthogonal confirmation and further validation on non-pooled sample sets. A fucosylated version of AFP is well described in sera associated with liver cancers (39,40). Identification of an AFP variant in prostate cancer sera is a novel finding, while identification of the haptoglobin and ApoA-I variants are also consistent with previous expression profiling studies of cancer sera (32, 64-67). These glycoprotein isoforms may represent new potential biomarkers for detection of disease, monitoring cancer treatment, or surveillance for recurrence post-therapy. Regardless of the eventual disposition of

these examples as true biomarkers, this exercise demonstrates the ability to achieve disease-group specific differential lectin capture.

We next examined the effects of two serum/plasma protein depletion strategies on the types of serum glycoproteins captured by lectins. As a comparison we show a representative display of the glycoproteins that bind SNA1 lectin versus serum proteins that do not bind SNA1 lectin (Figure 3 lanes 1 and 2). For the six lectins tested under the conditions utilized, the lack of albumin binding to the lectins has been consistent. This lack of albumin binding to lectins is a clear advantage to their use as biological affinity reagents and for up-front fractionation strategies. Indeed, the lectin-capture step may simultaneously concentrate classes of glycoproteins and eliminate the major blood proteins. To evaluate depletion of major blood proteins prior to lectin incubation, two kits were used with a PCa cancer sera pool: a ProteoPrep 20 Plasma Immunodepletion Kit (Sigma-Aldrich), which uses antibody capture to remove 20 of the most prevalent blood proteins, and a Montage Albumin Deplete Kit (Millipore), which uses a blue-dye affinity resin. Under the conditions used, both kits were very effective at removing major serum proteins (Figure 3, lanes 8 and 9). For comparison, an aliquot of unfractionated serum is shown (Figure 3, lane 10). The depleted protein fraction was then incubated with SNA1 lectin, and the bound (Figure 3, lanes 4 and 6) and unbound proteins (Figure 3, lanes 5 and 7) separated by SDS-PAGE. Comparison of the bound protein profiles from the depeleted fractions with that of the lectin alone indicate a highly similar pattern of bound proteins, independent of depletion. Similar results were obtained with the AAL, ConA and WGA lectins (not shown). Much additional quantitative evaluation of these fractions remains to be done, but these results demonstrate that lectins can serve as excellent initial

fractionation resins for enrichment of serum glycoproteins, both with and without pre-depletion.

Fucosylation changes in serum proteins associated with HCC carcinogenesis

The best documented change that occurs in glycosylation during the development of hepatocellular carcinoma (HCC) is an increase in the level of core α-1,6 linked fucosylation of AFP (39,40). In HCC and in testicular cancer, the glycosylation of AFP shifts from a simple biantennary glycan to an α-1,6 linked core fucosylated biantennary glycan. These changes have been observed by both direct glycan sequencing of AFP and by increased reactivity of AFP with a variety of lectins that preferentially bind to fucose containing glycan (41). The glycoform of AFP that reacts preferentially with the lectin *lens culinaris* (LCH) is referred to as AFP-L3, and it has been characterized as being a more specific marker of HCC than total AFP protein levels (68-70). Because of this, AFP-L3 was approved by the U.S Food and Drug Administration in 2005 to be the only diagnostic assay available for HCC.

Recent glycan analysis of whole serum (71), or serum that has been depleted of immunoglobulin (32,33), have reported that increases in the levels of core fucosylation of many serum glycoproteins was observed with the development of HCC. To identity those proteins that had increased fucosylation, the fucosylated glycoproteins associated with sera from either pooled normal or pooled HCC positive individuals were extracted using fucose specific lectins (LCH, AAA and AAL) and the proteome analyzed by either two dimensional gel electrophoresis (2DE) or by a simple LC MS/MS based methodology designed to identify fucosylated peptides (described in 33). A representative list of the

fucosylated proteins identified are provided in table 2. The levels of fucosylated glycoforms (Fc) of serum glycoproteins like Fc-α-1-acid glycoprotein, Fc-ceruloplasmin, Fc-alpha-2-macroglobulin, Fc-hemopexin, Fc-Apo-D, Fc-HBsAg, and Fc-Kininogen were increased in patients with HCC, while the levels of Fc-haptoglobin was decreased in the those patients (33). A similar methodology was utilized by us in an animal model of HBV induced HCC and identified a potential biomarker termed GP73 that has been shown to be 2-3 time more sensitive than AFP (32,72).

We have examined the level of Fc-GP73 and Fc-hemopexin in a small patient cohort containing a total of 80 patients with varying degrees of liver disease (n=20 each healthy subjects, HBV carriers, HBV cirrhosis, HCC). Analysis of these samples was performed for total GP73 level, for the level of fucosylated GP-73 (Fc-GP73), and for the level of fucosylated hemopexin (Fc-hemopexin). Total GP73 was analyzed by immunoblot using whole serum, and fucosylated species were analyzed by LCH lectin extraction of 5 µl of serum followed by immunoblotting of the fucosylated fraction as previously described (32,33). A representative blot from a subset of these samples, and the effects on the sensitivity, specificity, and positive predicative values for these three markers are presented in figure X. Use of total GP73 levels alone had a sensitivity of 65% and a specificity of 90%, which was very similar to our larger blinded study using this marker (72). If the fucosylated glycoforms, Fc-GP73 and Fc-hemopexin, are evaluated alone, this improves the sensitivities to $\geq$ 90% and specificities of 100%. These results are only from a small sample set, and larger studies are ongoing, but it demonstrates that specific fucosylated isoforms of serum glycoproteins represent a rich pool of new biomarker targets for HCC, and likely many other cancers.

Automatation of lectin-capture for mass spectrometry-based protein expression profiling.

One of our goals in developing the lectin capture strategies was to incorporate this into a relatively high-throughput serum expression profiling platform. This would facilitate analysis of the large number of clinical samples necessary to accommodate the many disease variables associated with cancers, as well as equally important epidemiological, study design and biostatistical issues. One approach is summarized in figure 1. This involves capture of the intact glycoproteins by lectins, followed by trypsin digestion to generate peptides and final affinity separation of peptides prior to MALDI-TOF and/or tandem mass spectrometry analyses. All of these steps can be fully automated using bead-based supports coupled to liquid sample handling robotics. Commercially available configurations to accomplish this are already available, typified by the Bruker Daltonics ClinProt® system (9,62), which we have used for our analyses. Shown in figure 5 is an example of this approach as applied to pooled sera from biopsy proven early prostate cancer and benign disease, with PSA values for both sets ranging from 2-4 ng/ml. In the left panel, a gel image of ConA bound serum glycoproteins before and after trypsin digestion is shown. An aliquot of the undigested and digested proteins were incubated with IMAC-Cu magnetic beads, processed robotically, and eluted peptides/proteins spotted on a steel plate for MALDI-TOF analysis. In the right panel we show a comparison of the MALDI-TOF spectra of the two fractions, with or without trypsin digestion. The increased detection of m/z peaks in the trypsin digested samples is consistent with assessment of high molecular weight proteins that are not well resolved as whole proteins. In addition, we can observe specific differences in the spectra between

PCa and BPH. On a larger scale, we propose that this approach can be used to generate spectra for expression profiling purposes and bioinformatics analyses, and also serve as targets for further tandem MS analysis. In our specific example, this would be done using automated MS/MS using the MALDI-TOF/TOF. However, we have also used this approach successfully when coupled with differential labeling (iTRAQ) and analysis using ESI-MS/MS. In point of fact, the lectin affinity-capture of whole proteins should be amenable to most mass spectrometry based analysis.

A Pre-Validation Strategy for Quantitative Assessment of Protein Biomarker Isoforms.

We have demonstrated that lectin-based fractionation strategies can be an excellent initial front-end step for serum glycoprotein isolation and a powerful approach toward biomarker discovery. However, establishment of intial discovery approaches should be complimented with compatible analytical confirmation and pre-validation strategies. For this purpose we have recommended targeted affinity methods sometimes referred to as immuno-MS (73, 74) The targeted candidate biomarker may be either specific isoforms of whole proteins or subunits and fragments of larger proteins. We recently demonstrated the power of immuno-MS in the characterization of an isoform of ApoA-II, the over-expression of which was confirmed to be specific to benign prostate hyperplasia (BPH) and/or PCa as compared to healthy controls (73). Interestingly, although ApoA-II is known to exist as an 8.7 kD protein in serum, we found that a unique isoform with a mass of 8.9 kD was associated with disease state. The individual serum isoforms of ApoA-II can be revealed by immuno-capture and mass spectrometry analysis whereas standard immuno-assay techniques cannot generally discriminate isoforms. In

figure 6 we show the specific capture of this unique isoform of serum ApoA-II from disease specimens only. However, even though we could easily capture purified ApoA-II, we were unable to capture significant amounts of wild-type ApoA-II from serum. Thus, combining immuno-capture with mass spectrometry allows for analysis of specific and altered forms of the target protein and is a useful intermediate step toward the maturation of a biomarker from discovery to utility.

Clearly, such targeted proteomic strategies that utilize high-affinity or baiting strategies can be used to selectively enrich lower abundance proteins. Baiting strategies can also be used to identify biomarker function and to associate the biomarker with a disease pathway through identification of other proteins with which it interacts. This technology may also bridge the gap between hypothesis- and data-driven biomarker discovery by allowing functional baiting of entire classes of biomarkers that are implicated in disease and disease progression. An affinity pre-enrichment of target proteins has two advantages: low abundance proteins can be seen and quantified, and the identity of the proteins (their primary sequence) is established, allowing subsequent isoform characterization. We have begun using a recently developed technology, isotope-differentiated binding energy shift tags (IDBEST™, Target Discovery, Inc.)(43,75), to quantitate specific serum isoforms, including glycoforms. IDBEST™ uses specific tags which exploit the natural phenomenon of mass defect. The mass defect is related to the nuclear binding energy released upon formation and stabilization of the nucleus of a given element. Bio-molecules have a very negligible mass shift. A maximum mass defect value of ~0.1 amu is obtained for elements with atomic numbers between 35(Br) and 63(Eu). These isotope-differentiated binding-energy–shift tags shift the peaks of all the

tagged species by about 0.1 Da, allowing software to discriminate tagged from untagged species directly in the mass spectrum and thus eliminating the need for affinity cleanup of the tagged samples.

As an example of the application of this technique to biomarker assessment we labeled a target and reference samples and then followed with immuno-capture of PSA. The specifically isolated protein complexes are trypsin digested and subjected to MALDI-TOF analysis. In figure 7 we show an example of the specific quantitation of a PSA peptide using this approach. We are currently evaluating the quantization accuracy of this approach by parallel sampling against clinical PSA information. We anticipate using the IDBEST™ technology for the quantitation of specific isoforms of known biomarkers in prostate cancer, such as PSA, as well isoforms of apolipo-proteins. Specific peptides that encompass structural changes (isoforms) of whole proteins can be targeted by modification of the isolation process. Thus, for glycoforms we would pre-treat with glycosidase to remove the glycan and identify the peptide via resulting mass shift. The ability to carry early discovery through confirmation and rapid pre-validation, prior to the expensive and time-consuming process of developing isoform or glycospecific antibodies, should prove advantageous to accelerating biomarker discovery.

Summary

The continued improvements in proteomic mass spectrometry technologies, coupled with the human and other genome databases, has allowed unprecedented opportunities for biomarker protein discovery and analysis of complex proteomes like serum. An underlying issue will always be the quality of the starting material, as this will

ultimately dictate the quality of the proteomic data and utility of this for clinical purposes. In this regard, sample collection, storage and quality issues, epidemiological input and study design biases will always influence clinical proteomic studies. Building from what was learned in the first wave of serum protein expression profiling studies, strategies striking a balance of sample throughput with improved depth of serum protein capture and protein identification needs to continue to evolve. Accommodating these concerns into proteomic analysis design is facilitated by use of the lectins as front-end fractionation and enrichment tools. We have only described the use of six lectins, and there are dozens of other individual lectin types available commercially that remain to be empirically assessed, and hundreds more described in the literature (21,45). Additionally, serial affinity capture strategies in which different lectins are used in tandem will increase the fractionation capabilities beyond the discussed examples. The use of a biological affinity approach targeting known glycosylation changes associated with cancer is an additional benefit. Coupling the glycoprotein characterizations to some type of simultaneous or complementary glycan analysis of the same samples will further extend the utility of this approach. Identification of altered carbohydrate content, whether it is sialic acid or fucose residue differences, automatically implicates the corresponding glycosyltransferases or (glycosidases) as potential participants in the oncogenesis of a particular cancer subtype. These enzyme classes can readily be assessed by other methods than proteomics, including gene or tissue microarrays, and basic biochemical enzymatic assays.

LITERATURE CITED

1. Adam, B.L., Qu, Y., Davis, J.W., Ward, M.D., Clements, M.A., Cazares, L.H., Semmes, O.J., Schellhammer, P.F., Yasui, Y., Feng, Z. and Wright, G.L., Jr. (2002) Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. *Cancer Res.* 62, 3609-3614.

2. Conrads, T.P., Hood, B.L., Issaq, H.J. and Veenstra, T.D. (2004) Proteomic patterns as a diagnostic tool for early-stage cancer: a review of its progress to a clinically relevant tool. *Mol. Diagn.* 8, 77-85.

3. Campa, M.J., Wang, M.Z., Howard, B., Fitzgerald, M.C. and Patz, E.F., Jr. (2003) Protein expression profiling identifies macrophage migration inhibitory factor and cyclophilin a as potential molecular targets in non-small cell lung cancer. *Cancer Res.* 63, 1652-1656.

4. Yanagisawa, K., Shyr, Y., Xu, B.J., Massion, P.P., Larsen, P.H., White, B.C., Roberts, J.R., Edgerton, M., Gonzalez, A., Nadaf, S., Moore, J.H., Caprioli, R.M. and Carbone, D.P. (2003) Proteomic patterns of tumour subsets in non-small-cell lung cancer.
Lancet 362, 433-439.

5. Semmes, O.J. (2005) The "omics" haystack: defining sources of sample bias in expression profiling. *Clin. Chem.* 51, 1571-1572.

6. Semmes, O.J., Malik, G. and Ward, M. (2006) Application of mass spectrometry to the discovery of biomarkers for detection of prostate cancer. *J. Cell Biochem.* 98, 496-503.

7. Wright GL, Jr. (2002) SELDI proteinchip MS: a platform for biomarker discovery and cancer diagnosis. *Expert Rev. Mol. Diagn.* 2, 549-563.

8. Villanueva, J., Philip, J., Entenberg, D., Chaparro, C.A., Tanwar, M.K., Holland, E.C. and Tempst, P. (2004) Serum peptide profiling by magnetic particle-assisted, automated sample processing and MALDI-TOF mass spectrometry. *Anal. Chem.* 76, 1560-1570.

9. Pusch, W. and Kostrzewa, M. (2005) Application of MALDI-TOF mass spectrometry in screening and diagnostic research. *Curr. Pharm. Des*. 11, 2577-2591.

10. Baggerly, K.A., Morris, J.S. and Coombes, K.R. (2004) Reproducibility of SELDI-TOF protein patterns in serum: comparing datasets from different experiments. *Bioinformatics* 20, 777-785.

11. Ransohoff, D.F. (2005) Lessons from controversy: ovarian cancer screening and serum proteomics. *J. Natl. Cancer Inst.* 97, 315-319.

12. Sorace, J.M. and Zhan, M. (2003) A data review and re-assessment of ovarian cancer serum proteomic profiling. *BMC Bioinformatics* 4, 24.

13. Hu, J., Coombes, K.R., Morris, J.S. and Baggerly, K.A. (2005) The importance of experimental design in proteomic mass spectrometry experiments: some cautionary tales. *Brief Funct. Genomic Proteomic* 3, 322-331.

14. Ransohoff, D.F. (2005) Bias as a threat to the validity of cancer molecular-marker research. *Nat. Rev. Cancer* 5, 142-149.

15. Semmes, O. J., Feng, Z., Adam, B.L., Banez, L.L., Bigbee, W.L., Campos, D., Cazares, L.H., Chan, D.W., Grizzle, W.E., Izbicka, E., Kagan, J., Malik, G., McLerran, D., Moul, J.W., Partin, A., Prasanna, P., Rosenzweig, J., Sokoll, L.J., Srivastava, S., Srivastava, S., Thompson, I., Welsh, M.J., White, N., Winget, M., Yasui, Y., Zhang, Z., and Zhu, L. (2005) Evaluation of serum protein profiling by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry for the detection of prostate cancer: I. Assessment of platform reproducibility. *Clin. Chem.* 51, 102-112.

16. Caprioli, R.M. (2005) Deciphering protein molecular signatures in cancer tissues to aid in diagnosis, prognosis, and therapy. *Cancer Res.* 65, 10642-10645.

17. Li, J., Orlandi, R., White, C.N., Rosenzweig, J., Zhao, J., Seregni, E., Morelli, D., Yu, Y., Meng, X.Y., Zhang, Z., Davidson, N.E., Fung, E.T. and Chan, D.W. (2002) Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. *Clin. Chem.* 48, 1296-1304.

18. Hakomori, S. (1996) Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res.*, 56, 5309-5318.

19. Kobata, A. (1998) A retrospective and prospective view of glycopathology. *Glycoconj. J.* 15, 323-331.

20. Butler, M., Quelhas, D., Critchley, A.J., Carchon, H., Hebestreit, H.F., Hibbert, R.G., Vilarinho, L., Teles, E., Matthijs, G., Schollen, E., Argibay, P., Harvey, D.J., Dwek, R.A., Jaeken, J. and Rudd, P.M. (2003) Detailed glycan analysis of serum glycoproteins of patients with congenital disorders of glycosylation indicates the specific defective glycan processing step and provides an insight into pathogenesis. *Glycobiology* 13, 601-622.

21. Sharon, N. and Lis, H. (2004) History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology* 14, 53R-62R.

22. Kim, Y.J. and Varki, A. (1997) Perspectives on the significance of altered glycosylation of glycoproteins in cancer. *Glycoconj. J.* 14, 569-576.

23. Dennis, J.W., Granovsky, M., and Warren, C.E. (1999) Protein glycosylation in development and disease. *BioEssays* 21, 412–421.

24. Orntoft, T.F. and Vestergaard, E.M. (1999) Clinical aspects of altered glycosylation of glycoproteins in cancer. *Electrophoresis* 20, 362-371.

25. Fernandes, B., Sagman, U., Auger, M., Demetrio, M. and Dennis, J.W. (1991) Beta 1,6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. *Cancer Res.* 51, 718-723.

26. Yanagi, M., Aoyagi, Y., Suda, T., Mita, Y. and Asakura, H. (2001) N-Acetylglucosaminyltransferase V as a possible aid for the evaluation of tumor invasiveness in patients with hepatocellular carcinoma. *J. Gastroenterol. Hepatol*. 16, 1282-1289.

27. Siddiqui, S.F,, Pawelek, J., Handerson, T., Lin, C.Y., Dickson, R.B., Rimm, D.L. and Camp, R.L. (2005) Coexpression of beta1,6-N-acetylglucosaminyltransferase V glycoprotein substrates defines aggressive breast cancers with poor outcome. *Cancer Epidemiol. Biomarkers Prev.* 14, 2517-2523.

28. Handerson, T., Camp, R. Harigopal, M., Rimm, D. and Pawelek, J. (2005) Beta 1,6-Branched oligosaccharides are increased in lymph node metastases and predict poor outcome in breast carcinoma. *Clin. Cancer Res.* 11, 2969-2973.

29. Dwek, M.V., Lacey, H.A. and Leathem, A.J. (1998) Breast cancer progression is associated with a reduction in the diversity of sialylated and neutral oligosaccharides. *Clin. Chim. Acta* 271, 191-202.

30. Dwek, M.V., Ross, H.A. and Leathem, A.J. (2001) Proteome and glycosylation mapping identifies post-translational modifications associated with aggressive breast cancer. *Proteomics* 1, 756-762.

31. Peracaula, R., Tabares, G., Royle, L., Harvey, D.J., Dwek, R.A., Rudd, P.M. and de Llorens, R. (2003) Altered glycosylation pattern allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. *Glycobiology* 13, 457-470.

32. Block, T. M., Comunale, M. A., Lowman, M., Steel, L. F., Romano, P. R., Fimmel, C., Tennant, B. C., London, W. T., Evans, A. A., Blumberg, B. S., Dwek, R. A., Mattu, T. S. and Mehta, A. S. (2005) Use of targeted glycoproteomics to identify serum glycoproteins that correlate with liver cancer in woodchucks and humans. *Proc Natl Acad Sci U S A* 102, 779-784.

33. Comunale, M.A., Lowman. M., Long, R.E., Krakover, J., Philip, R., Seeholzer, S., Evans, A.A., Hann, H.W., Block, T.M. and Mehta, A.S. (2006) Proteomic analysis of serum associated fucosylated glycoproteins in the development of primary hepatocellular carcinoma. *J. Proteome Res.*5, 308-315.

34. Prakash, S. and Robbins, P.W. (2000) Glycotyping of prostate specific antigen. *Glycobiology*, 10, 173-176.

35. Ohyama, C., Hosono, M., Nitta, K., Oh-eda, M., Yoshikawa, K., Habuchi, T., Arai, Y., and Fukuda, M. (2004) Carbohydrate structure and differential binding of prostate specific antigen to Maackia amurensis lectin between prostate cancer and benign prostate hypertrophy. *Glycobiology* 14, 671–679.

36. Tabares, G., Radcliffe, C.M., Barrabes, S., Ramirez, M., Aleixandre, R.N., Hoesel, W., Dwek, R.A., Rudd, P.M., Peracaula, R. and de Llorens, R. (2006) Different glycan structures in prostate-specific antigen from prostate cancer sera in relation to seminal plasma PSA. *Glycobiology* 16, 132-145.

37. Ward, M.A., Catto, J.W.F., and Hamdy, F.C. (2001) Prostate-specific antigen: biology, biochemistry and available commercial assays. *Ann. Clin. Biochem.* 38, 633–651.

38. Diamandis, E. (1998) Prostate-specific antigen: its usefulness in clinical medicine. *Trends Endocrinol. Metab.* 9, 310–316.

39. Miyazaki, J., Endo, Y. and Oda, T. (1981) Lectin affinities of alpha-fetoprotein in liver cirrhosis, hepatocellular carcinoma and metastatic liver tumor. *Acta Hepatol. Jpn*. 22, 1559-1568.

40. Breborowicz, J., Mackiewicz, A. and Breborowicz, D. (1981) Microheterogeneity of alpha-fetoprotein in patient serum as demonstrated by lectin affino-electrophoresis. *Scand. J. Immunol*. 14, 15-20.

41. Johnson, P. J., Poon, T. C., Hjelm, N. M., Ho, C. S., Blake, C. and Ho, S. K. (2000). Structures of disease-specific serum alpha-fetoprotein isoforms. *Br. J. Cancer* 83, 1330-1337.

42. Xiong, L., Andrews, D. and Regnier, F. (2003) Comparative proteomics of glycoproteins based on lectin selection and isotope coding. *J. Proteome Res.* 2, 618-625.

43. Hall, M.P. and Schneider, L.V. (2004) Isotope-differentiated binding energy shift tags (IDBEST) for improved targeted biomarker discovery and validation. *Exp. Rev. Proteomics* 1, 421-431.

44. Goldstein, I.J. (2002) Lectin structure-activity: the story is never over. *J. Agric. Food Chem.* 50, 6583-6585.

45. Hirabayashi, J. (2004) Lectin-based structural glycomics: glycoproteomics and glycan profiling. *Glycoconj J.* 21, 35-40.

46. Lis, H. and Sharon, N. (1998) Lectins: Carbohydrate-Specific Proteins That Mediate Cellular Recognition. *Chem. Rev*. 98, 637–674.

47. Dam, T.K., Roy, R., Das, S.K., Oscarson, S. and Brewer, C.F. (2000) Binding of multivalent carbohydrates to concanavalin A and Dioclea grandiflora lectin: Thermodynamic analysis of the "multivalency effect." *J. Biol. Chem.* 275, 14223-14230.

48. Mandal, D. K., Kishore, N. and Brewer, C. F. (1994) Thermodynamics of lectin-carbohydrate interactions: Titration microcalorimetry measurements of the binding of N-linked carbohydrates and ovalbumin to concanavalin A. *Biochemistry* 33, 1149–1156.

49. Alvarez-Manilla, G., Atwood, J. 3rd, Guo, Y., Warren, N.L., Orlando, R. and Pierce, M. (2006) Tools for glycoproteomic analysis: size exclusion chromatography facilitates identification of tryptic glycopeptides with N-linked glycosylation sites. *J. Proteome Res*. 5, 701-708.

50. Qiu, R. and Regnier, F.E. (2005) Comparative glycoproteomics of N-linked complex-type glycoforms containing sialic acid in human serum. *Anal Chem.* 77, 7225-7231.

51. Zhang, H., Li, X.J., Martin, D.B. and Aebersold, R. (2003) Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol*. 21, 660-666.

52. Wright, M.E., Han, D.K. and Aebersold, R. (2005) Mass spectrometry-based expression profiling of clinical prostate cancer. *Mol. Cell Proteomics* 4, 545-554.

53. Yang, Z., Hancock, W.S., Chew, T.R. and Bonilla,L. (2005) A study of glycoproteins in human serum and plasma reference standards (HUPO) using multilectin affinity chromatography coupled with RPLC-MS/MS. *Proteomics* 5, 3353-3366.

54. Liu, T., Qian, W.J., Gritsenko, M.A., Camp, D.G. 2nd, Monroe, M.E., Moore, R.J. and Smith, R.D. (2005) Human Plasma N-Glycoproteome Analysis by Immunoaffinity Subtraction, Hydrazide Chemistry, and Mass Spectrometry. *J. Proteome Res.*, 4, 2070 -2080

55. Wang, Y., Wu, S.L. and Hancock, W.S. (2006) Approaches to the Study of N-linked Glycoproteins in Human Plasma Using Lectin Affinity Chromatography and Nano-HPLC Coupled to Electrospray Linear Ion Trap - Fourier Transform Mass Spectrometry (LTQ/FTMS). *Glycobiology,* In press.

56. Itoh, S., Kawasaki, N., Hashii, N., Harazono, A., Matsuishi, Y., Hayakawa, T. and Kawanishi, T. (2006) N-linked oligosaccharide analysis of rat brain Thy-1 by liquid chromatography with graphitized carbon column/ion trap-Fourier transform ion cyclotron resonance mass spectrometry in positive and negative ion modes. *J.Chromatogr. A* 1103, 296-306

57. Mao, X., Luo, Y., Dai, Z., Wang, K., Du, Y. and Lin, B. (2004).Integrated lectin affinity microfluidic chip for glycoform separation. *Anal. Chem.* 76, 6941-6947.

58. Kuno, A., Uchiyama, N., Koseki-Kuno, S., Ebe, Y., Takashima, S., Yamada, M. and Hirabayashi, J. (2005) Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. *Nat. Methods*. 2, 851-856.

59. Comelli, E.M., Head, S.R., Gilmartin, T., Whisenant, T., Haslam, S.M., North, S.J., Wong, N.K., Kudo, T., Narimatsu, H., Esko, J.D., Drickamer, K., Dell, A. and Paulson, J.C. (2006) A focused microarray approach to functional glycomics: transcriptional regulation of the glycome. *Glycobiology* 16, 117-131.

60. Novotny, M.V. and Mechref, Y. (2005) New hyphenated methodologies in high-sensitivity glycoprotein analysis. *J. Sep. Sci.* 28, 1956-1968.

61. Fromell, K., Andersson, M., Elihn, K. and Caldwell, K.D. (2005) Nanoparticle decorated surfaces with potential use in glycosylation analysis. *Colloids Surf. B Biointerfaces* 46, 84-91

62. Sparbier, K., Koch, S., Kessler, I., Wenzel, T. and Kostrzewa, M. (2005) Selective isolation of glycoproteins and glycopeptides for MALDI-TOF MS detection supported by magnetic particles. *J. Biomol. Tech.* 16, 407-413.

63. Comunale, M.A., Mattu, T.S., Lowman, M.A., Evans, A.A., London, W.T., Semmes, O.J., Ward, M., Drake, R.R., Romano, P.R,, Steel, L.F., Block, T.M. and Mehta, A. (2004) Comparative proteomic analysis of de-N- glycosylated serum from hepatitis B carriers reveals polypeptides that correlate with disease status. *Proteomics* 4, 826-838.

64. Zhang, Z., Bast, R.C. Jr,, Yu, Y., Li, J., Sokoll, L.J., Rai, A.J., Rosenzweig, J.M., Cameron, B., Wang, Y.Y., Meng, X.Y., Berchuck, A., Van Haaften-Day, C., Hacker, N.F., de Bruijn, H.W., van der Zee, A.G., Jacobs, I.J., Fung, E.T. and Chan, D.W. (2004) Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res.* 64, 5882-5890.

65. Semmes, O.J., Cazares, L.H., Ward, M.D., Qi, L., Moody, M., Maloney, E., Morris, J., Trosset, M.W., Hisada, M., Gygi, S. and Jacobson, S. (2005) Discrete serum protein signatures discriminate between human retrovirus-associated hematologic and neurologic disease. *Leukemia* 19, 1229-1238.

66. Wilson, N.L., Schulz, B.L., Karlsson, N.G. and Packer, N.H. (2002) Sequential analysis of N- and O-linked glycosylation of 2D-PAGE separated glycoproteins. *J. Proteome Res.* 1, 521-529.

67. Jin, Y. and Manabe, T. (2005) Direct targeting of human plasma for matrix-assisted laser desorption/ionization and analysis of plasma proteins by time of flight-mass spectrometry. *Electrophoresis* 26, 2823-2834.

68. Shiraki, K., K. Takase, Y. Tameda, M. Hamada, Y. Kosaka, and T. Nakano, (1995) A clinical study of lectin-reactive alpha-fetoprotein as an early indicator of hepatocellular carcinoma in the follow-up of cirrhotic patients. *Hepatology* 22, 802-807.

69. Taketa, K., C. Sekiya, M. Namiki, K. Akamatsu, Y. Ohta, Y. Endo, and K. Kosaka (1990) Lectin-reactive profiles of alpha-fetoprotein characterizing hepatocellular carcinoma and related conditions. *Gastroenterology* 99, 508-518.

70. Taketa, K., Ichikawa, E., Taga, H. and Hirai, H. (1985) Antibody-affinity blotting, a sensitive technique for the detection of alpha-fetoprotein separated by lectin affinity electrophoresis in agarose gels. *Electrophoresis* 6, 492-497.

71. Callewaert, N., Van Vlierberghe, H., Van Hecke, A., Laroy, W., Delanghe, J. and Contreras, R. (2004) Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics *Nature Med.* 10, 429-434.

72. Marrero, J. A., Romano, P. R., Nikolaeva, O., Steel, L., Mehta, A., Fimmel, C. J., Comunale, M. A., D'Amelio, A., Lok, A. S. and Block, T. M. (2005) GP73, a resident Golgi glycoprotein, is a novel serum marker for Hepatocellular Carcinoma. *J. Hepatol.* 43, 1007-1012.

73. Malik, G., Ward, M.D., Gupta, S.K., Trosset, M.W., Grizzle, W.E., Adam, B.L., Diaz, J.I. and Semmes, O.J. (2005) Serum levels of an isoform of apolipoprotein A-II as a potential marker for prostate cancer. *Clin. Cancer Res.* 11, 1073-1085.

74. Xiao, Z., Adam, B.L., Cazares, L.H., Clements, M.A., Davis, J.W., Schellhammer, P.F., Dalmasso, E.A. and Wright, G.L. Jr. (2001) Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. *Cancer Res.* 61, 6029-6033.

75. Hall, M.P., Ashrafi, S., Obegi, I., Petesch, R., Peterson, J.N. and Schneider, L.V. (2003)Mass defect" tags for biomolecular mass spectrometry. *J. Mass Spectrom.* 38, 809-816.

Figure Legends

Figure 1. Experimental flow chart combining lectin capture strategies with mass spectrometry approaches.

Figure 2. Differential Lectin Capture of Serum Glycoproteins. Pooled serum samples (30 ul) from benign prostatic hyperplasia and prostate cancer patients were incubated with the indicated lectin-agarose beads (50 ul bead volume) for 16 hrs (32, 63), and eluted with the respective monosaccharide. Bound proteins were separated on 8-16% Criterion SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA) and were visualized by silver staining.

Figure 3. Comparison of Serum Protein Depletion and Lectin Capture Strategies. A pooled prostate cancer serum pool (50 ul) was incubated with SNA1 lectin alone, or pre-fractionated with a Montage albumin depletion column (Millipore, Billerica, MA) or a ProteoPrep 20 column (Sigma-Aldrich, St. Louis, MO) prior to incubation with the SNA1 lectin. Eluted proteins from each column were separated on 8-16% SDS-gels and stained with Coomassie Blue as follows: 1. protein fraction not bound to SNA1; 2. protein fraction eluted from SNA1-agarose lectin beads; 3. serum protein fraction not bound to the Sigma ProteoPrep20 column; 4. ProteoPrep20-depleted proteins bound/eluted to SNA1; 5. ProteoPrep20 depleted serum protein fraction not bound to SNA1; 6. Montage-depleted serum protein fraction bound/eluted to SNA1; 7. Montage-depleted serum protein fraction not bound to SNA1; 8. Serum proteins bound/eluted to ProteoPrep 20 column; 9. Serum proteins bound to Montage column; 10. untreated serum.

Figure 4. The level of Fc-GP73 and Fc-hemopexin in the serum of patients with varying HCC disease states. A representative example of Fc-GP73 levels (A.) and Fc-hemopexin (B.) in normal (3 patients), cirrhotic (4 patients) and HCC+ (7 patients) as determined by immunoblot following isolation of fucosylated serum proteins. (C.) The sensitivity, specificity and positive predictive value of total GP73, Fc-GP73 and Fc-hemopexin in the patients shown in table 2.

Figure 5. Concanavalin A bound serum glycoproteins digested with trypsin prior to MALDI-TOF. Pooled serum from benign prostatic hyperplasia and prostate cancer sera were bound to concanavalin A lectin, then eluted. A portion of each eluate was separated on an 8-16% SDS-gel (A.), either intact (lanes 1,2) or digested overnight with trypsin (lanes 3,4). (B.) The same eluates, trypsin digested or intact, were incubated with IMAC-Cu magnetic beads, eluted and applied 1:1 with CHCA matrix to a steel plate for analysis on a Bruker Daltonics Ultraflex MALDI-TOF in linear mode.

Figure 6. Expression profile of ApoA-II isoforms in control and prostate cancer sera. Sera were processed on IMAC-Cu ProteinChips for SELDI-TOF MS as previously described (73). The mass region from 8000-9500 m/z is shown for purified ApoA-II (top spectra), sera from a prostate cancer patient (middle spectra) and sera from a healthy normal patient (bottom spectra).

Figure 7. Quantitative Immuno-MS assay for Prostate Specific Antigen. Purified prostate specific antigen was labeled with a lysine reactive ID-BEST™ mass defect reagent (Targeted Discovery, Inc., Palo Alto, CA), digested with trypsin, and analyzed on a Bruker Daltonics® UltraFlex™ MALDI-TOF/TOF. The raw spectra (top panel) and mass defect spectra (bottom panel) around the region of one of the expected PSA peptides is shown.

Table 1. Serum proteins identified after differential lectin capture

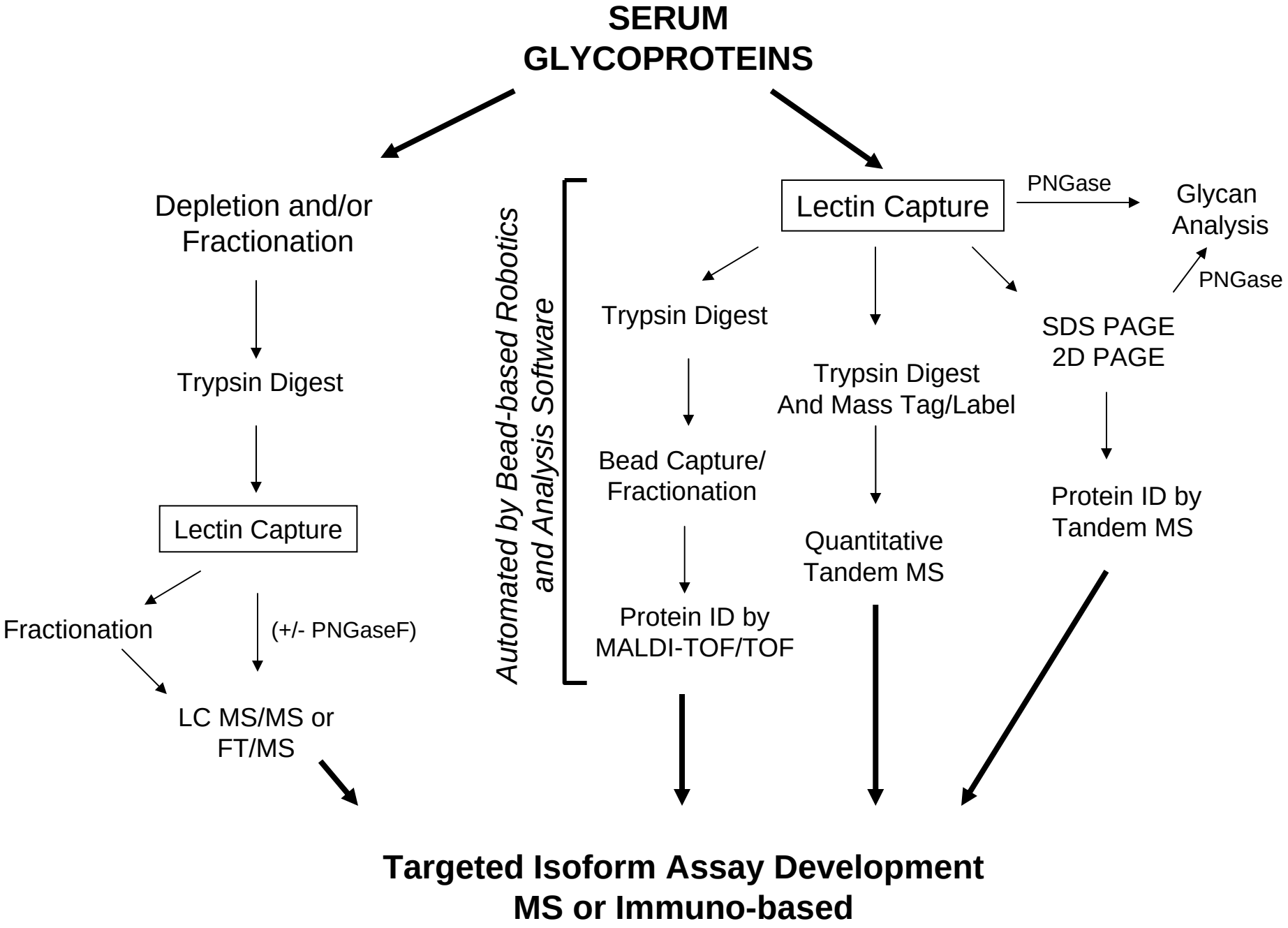
AAL	ConA
Albumin	Albumin
α–2–macroglobulin	α–2–macroglobulin
Immunoglobulin α Chain C	ApoA-1
Serotransferrin precursor	Serum amyloid P chain
AFP - alpha fetoprotein	Serotransferrin precursor
Haptoglobin	

AAA	SNA1
Albumin	α-1-antitrypsin
ApoA-1	ApoA-1
AFP - alpha fetoprotein	Complement factor B precursor
Myotubularin protein	Myotubularin related protein
Transthyretin precursor	Transferrin

Table 2. Proteins identified as altered in patients with HCC.

Identified Fucosylated Protein
Fc-GP-73
Fc-Hemopexin
Fc-HBsAg
Fc-AFP
Fc-alpha-acid-glycoprotein
Fc-alpha-1-antichymotrypsin
Fc-alpha-1-antitrypsin
Fc-Serotransferrin
Fc-Ceruloplasmin
Fc-alpha-2-macroglobulin
Fc-alpha-2-HS-glycoprotein (Fetuin A)
Fc-Haptoglobin
Fc-Fibrinogen gamma chain precursor
Fc-Complement factor B
Fc-IgG
Fc-IgA
Fc-APO-D
Fc-IgM
Fc-Kininogen
Fc-Histidine rch glycoprotein
Fc-Complement C1s component
Fc-alpha-1 B glycoprotein
Fc-B-2-glycoprotein (apo H)

Figure 1, MCP Drake et al.



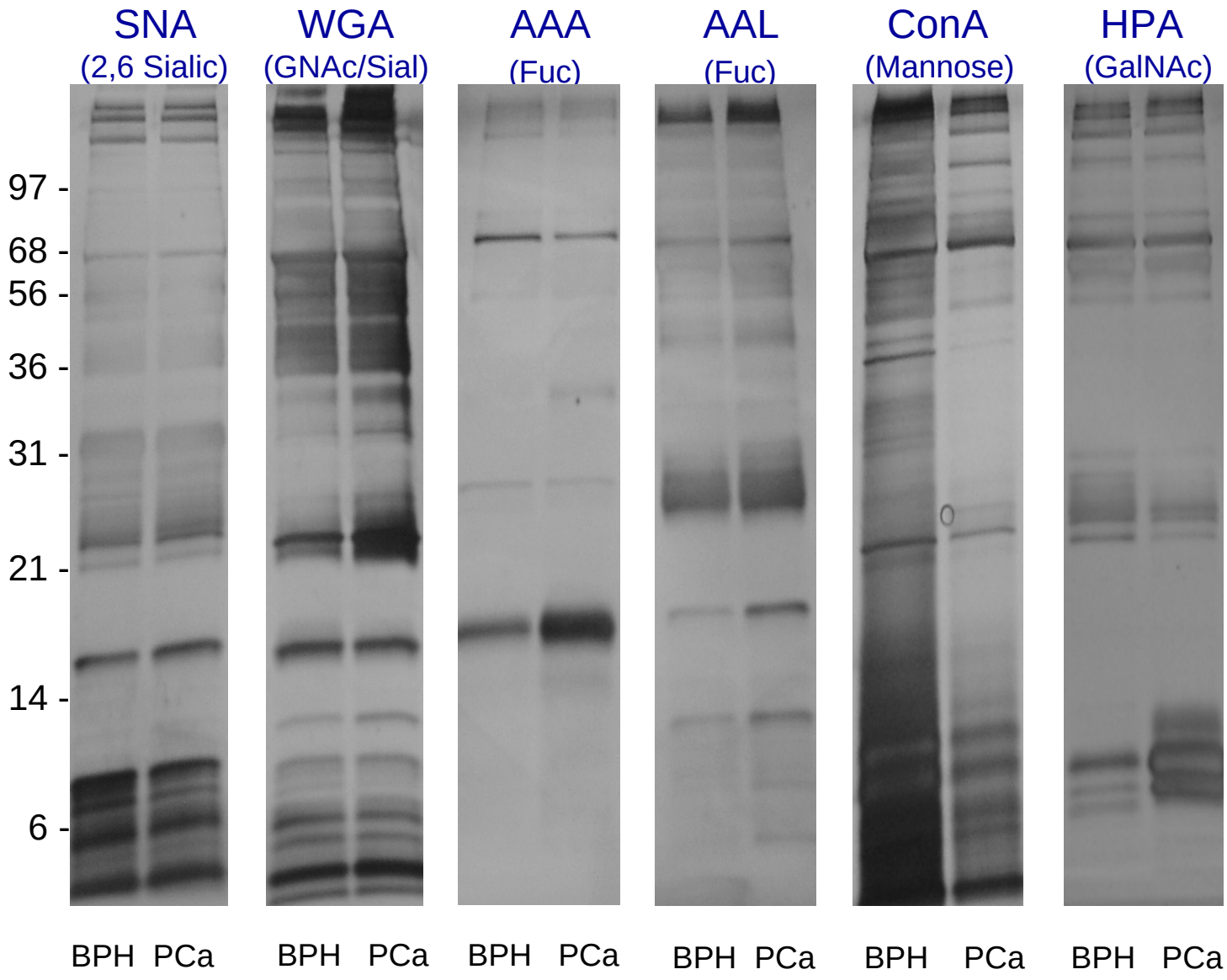


Figure 2, MCP Drake et al.

Figure 3, MCP Drake et al.

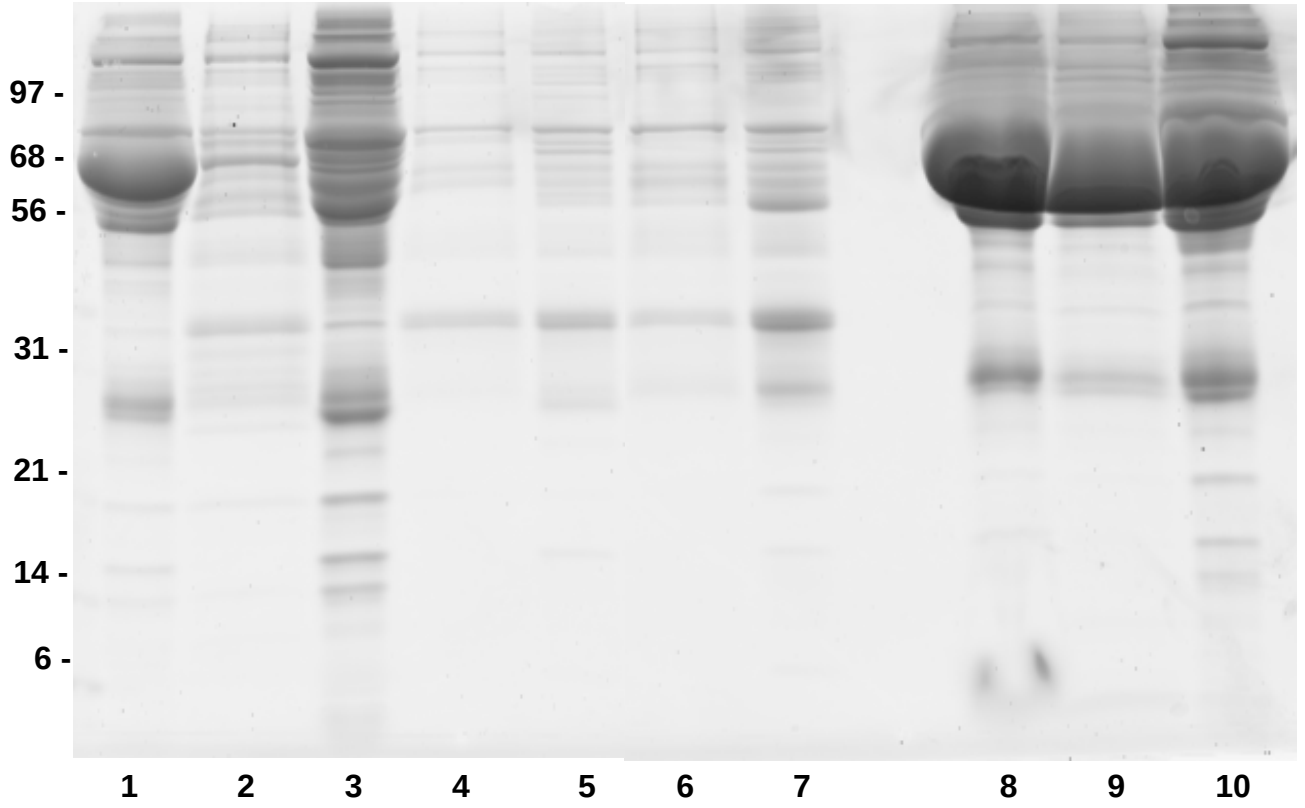
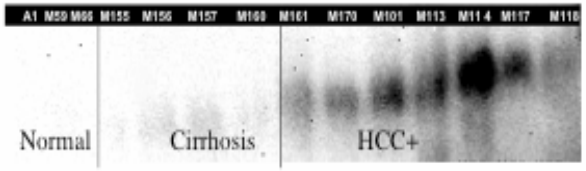


Figure 4, MCP Drake et al.

A



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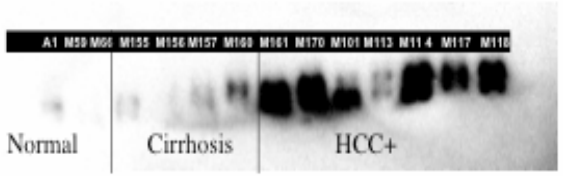

C

Marker	Sensitivity	Specificity	PPV
Total GP73	65%	90%	77%
FC-GP73	90%	100%	100%
Fc-hemopexin	95%	100	100%

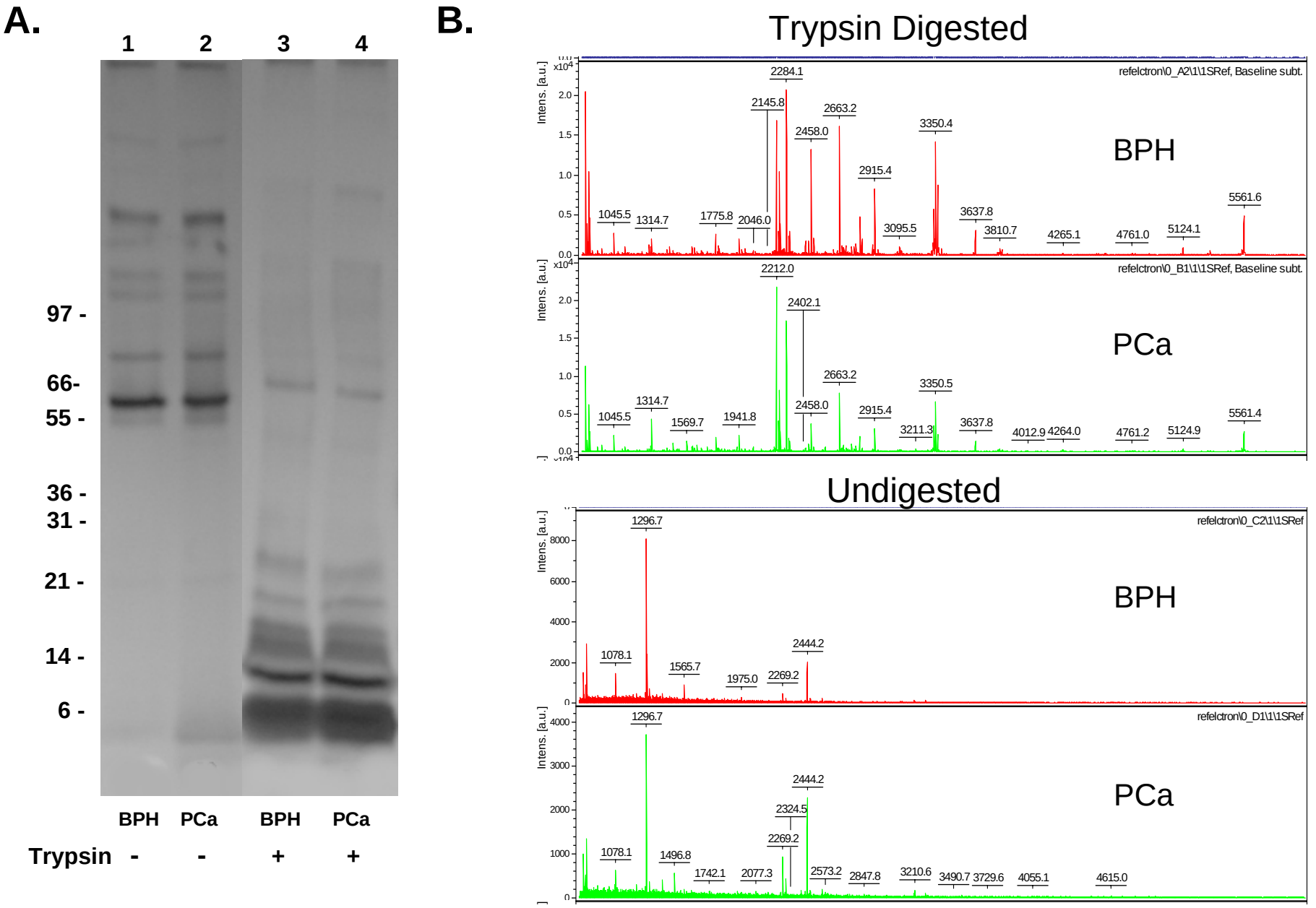

Figure 5, MCP Drake et al.

Figure 6, MCP Drake et al.

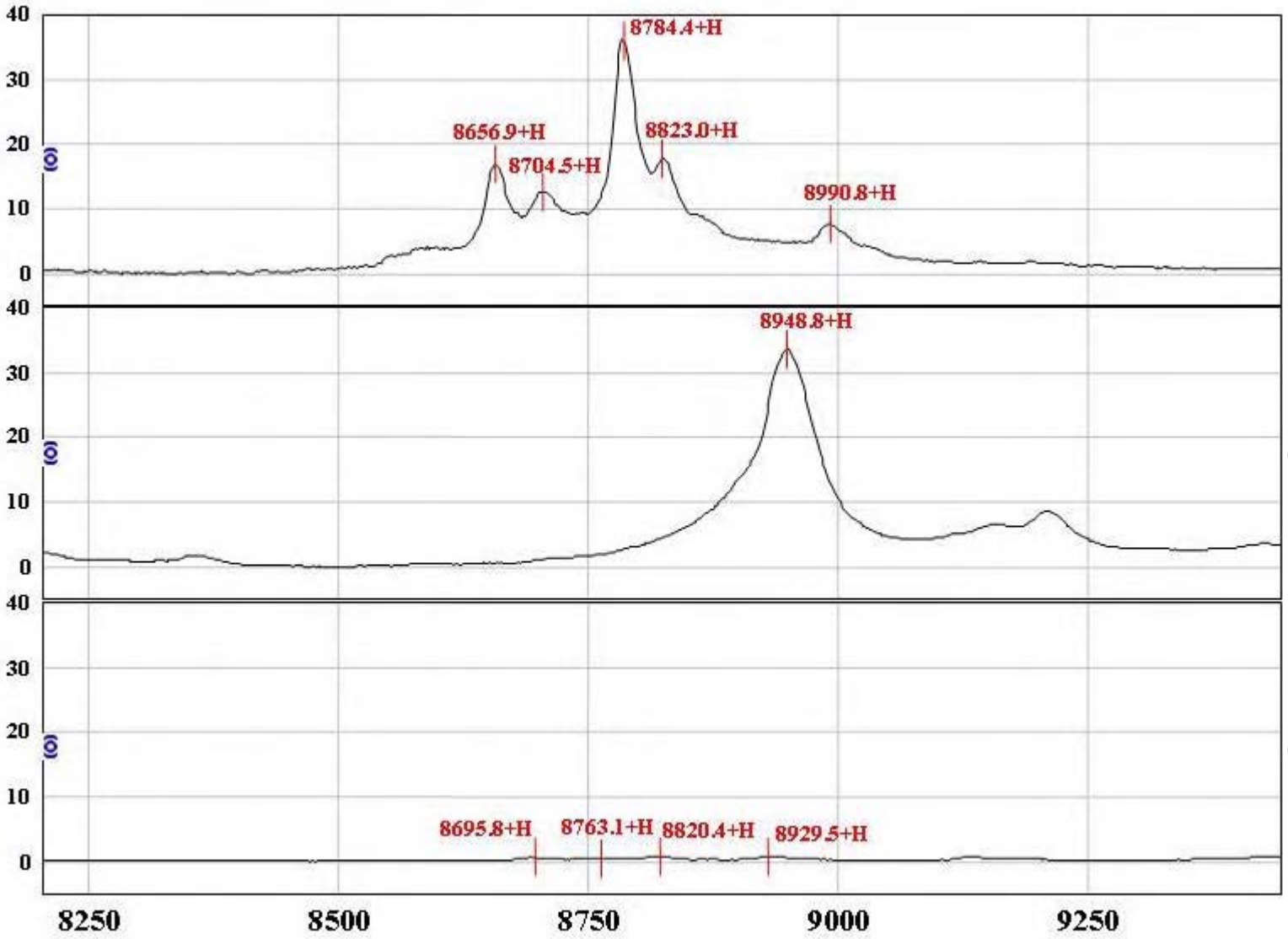

Figure 7, MCP Drake et al.